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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/005,202	12/04/2001	Keith D. Allen	R-902	6809

7590 10/27/2003

DELTAGEN, INC.
740 Bay Road
Redwood City, CA 94063

EXAMINER	
WILSON, MICHAEL C	

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 10/27/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/005,202	ALLEN, KEITH D.	
	Examiner	Art Unit	
	Michael C. Wilson	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *25 July 2003* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-28* is/are pending in the application.

4a) Of the above claim(s) *1,2,13 and 26-28* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *3-12 and 14-25* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) *06-03-02* .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Specification

New Fig. 3 and the amendment to the description of Fig. 3 have been entered.

The applications cited in the specification on pg 10, line 19, and pg 11, line 1, will need updated as necessary.

Election/Restrictions

Applicant's election without traverse of Group II, claims 3-12 and 14-25 is acknowledged.

The requirement is still deemed proper and is therefore made FINAL.

Claim 1, 2 and 26-28 are withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected invention, there being no allowable generic or linking claim.

Claims 3-12 and 14-25 are under consideration in the instant office action.

Claim Objections

Claim 9 is objected to because it is dependent upon claim 1 which is not under consideration.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 3-12 and 14-25 are rejected under 35 U.S.C. 101 because the claimed invention is not supported by either a specific or substantial asserted utility or a well-established utility.

Claims 6, 7 and 14-24 are directed toward a transgenic animal having a disruption of a Kir5.1 gene, an inwardly rectifying potassium channel. Claims 10 and 25 are directed toward methods of using the mice to identify compounds. The art at the time of filing did not teach mice with a disruption in the Kir5.1 gene. However, the art at the time of filing taught mice with a disruption in GIRK2 (Kir3.2) are indistinguishable from wild-type mice, while *wv/wv* mice, having a single point mutation in the Kir3.2 gene, had extensive cerebellar granule cell death, dopaminergic neuronal loss in the substantia nigra, male infertility, and spontaneous seizures (Signorini, 1997, PNAS, Vol. 94, pg 923-927). Thus, different mutations in inwardly rectifying potassium channels caused different phenotypes. The specification teaches making Kir5.1 -/- mice having dwarfed body shape (pg 53, lines 21-22), decreased body weight, spleen weight and spleen:body weight ratio (pg 54, lines 54), and increased startle response (pg 55, lines 8-11).

The mouse claimed does not have a specific utility. The specification suggests using the mice as a model of disease but does not disclose a specific disease in humans linked to a disruption in Kir5.1 (pg 18, lines 8-9; pg 19, lines 21-23). The specification suggests using the mice to compounds that alter a physiological response in the mice (pg 19, lines 5-20). The specification does not teach a disruption in Kir5.1 correlates to any specific disease or physiological response in humans, specifically

dwarfism, decreased spleen weight, or anxiety as claimed. Using the mice claimed to identify compounds is not specific to the mouse claimed because wild-type mice may be used to identify such compounds. In fact, any mouse can be used to find compounds that increase body weight, increase spleen weight or decrease the startle response. The specification teaches the "open field test" is generic to the hearing processing, sensory and motor processing, global sensory processing and motor abnormalities (pg 54, lines 20-25) as well as sensorimotor processing, attention, anxiety and thought disturbance (pg 54, lines 26-30); therefore, the "open field test" is not specific to any disease. Thus, using the mouse claimed to identify compounds is not specific to that mouse, and the mouse claimed does not have a use that is specific to any disease in humans.

The mouse claimed does not have a substantial utility. Claims 10-11, step c) require administering compounds to the mice and determining whether Kir5.1 gene expression is modulated. Compounds that modulate Kir5.1 expression cannot be found using the mice disclosed because Kir5.1 is not expressed in the mice. Claim 24 requires using identifying an agent that ameliorates a phenotype associated with Kir5.1 by administering compounds to the mice and determining whether a phenotype is ameliorated; however, the specification does not identify any compounds that alter physiological responses using the mice. Therefore, using the mouse to identify compounds is not substantial.

Claim 9 is included because it is directed toward making the mouse, which lacks utility for reasons above. Claims 3-5, 8 and 15, directed toward cells having a disrupted

Kir5.1 gene, and claims 11-12, directed toward using the cells to test compounds, are included because the cells lack a specific and substantial utility for the reasons above.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 3-12 and 14-25 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific or substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention.

In addition, the specification does not reasonably provide enablement for any animal, Kir5.1 gene, phenotype, cell, disruption, method of making a transgenic or method of using a transgenic as broadly claimed.

Claims 6, 7 and 14-24 are directed toward a transgenic animal having a disruption of a Kir5.1 gene. Claims 10 and 25 are directed toward methods of using the mice to identify compounds. The art at the time of filing did not teach mice with a disruption in the Kir5.1 gene. However, the art at the time of filing taught mice with a disruption in GIRK2 (Kir3.2) are indistinguishable from wild-type mice while *wv/wv* mice, having a single point mutation in the Kir3.2 gene, had extensive cerebellar granule cell death, dopaminergic neuronal loss in the substantia nigra, male infertility, and

spontaneous seizures (Signorini, 1997, PNAS, Vol. 94, pg 923-927). Thus, different mutations in inwardly rectifying potassium channels caused different results.

The specification teaches making Kir5.1 -/- mice having dwarfed body shape (pg 53, lines 21-22), decreased body weight, spleen weight and spleen:body weight ratio (pg 54, lines 54), and increased startle response (pg 55, lines 8-11).

The specification does not enable making or using a transgenic with a wild-type phenotype as encompassed by the claims. The transgenics throughout many of the claims do not recite any phenotype and may, therefore, have any phenotype including wild-type phenotype. The specification does not provide any use for a transgenic having a disruption in Kir5.1 that has a wild-type phenotype.

The specification does not teach how to make any cell having a disruption in a Kir5.1 (claims 3-5). Specifically, claims 4-5 encompass mice and rat cells. "Murine" encompasses mice and rats (http://www.m-w.com/cgi-bin/dictionary?book=Dictionary&va=murine). The only means of making a cell with a disruption in Kir5.1 taught in the specification is by using mouse embryonic stem cell technology. The state of the art at the time of filing was such that embryonic stem (ES) cell technology had only been successful in mice. Wagner (May 1995, Clin. and Experimental Hypertension, Vol. 17, pages 593-605) and Mullins (1996, J. Clin. Invest., Vol. 98, pages S37-S40) taught germline transmission of ES cells has not been demonstrated in species other than mice and the growth of ES cells from species other than mice is unreliable. Wall (1996, Theriogenology, Vol. 45, pg 57-68) taught transgene expression and the physiological result of such expression in livestock was

not always accurately predicted in transgenic mice (page 62, line 7). The specification fails to provide sufficient guidance to make transgenics other than mice by teaching obtaining ES cells in species other than mice. The specification does not teach the nucleic acid sequence of the Kir5.1 gene in non-mice, non-human species or correlate the Kir5.1 gene in mice to the Kir5.1 gene in other species. The specification does not teach how to make knockout animals other than mice or correlate making knockout mice to other species. Therefore, the specification does not provide adequate guidance for one of skill in the art to make cells having a disruption in Kir5.1 in any species other than mice.

Claim 9 is directed toward a method of making a transgenic mouse having a disruption in Kir5.1 using a cell having a construct with two sequences of Kir5.1, introducing the cell into a blastocyst, implanting the blastocyst into a pseudopregnant mouse which gives birth to chimeric mice, and breeding the chimeric mouse to produce the transgenic mouse. The claim does not require using mouse cells or an embryonic stem cell, which is considered essential to the invention. A mouse ES cell is the only type of cell taught in the specification that can be introduced into a blastocyst and result in a chimeric mouse as claimed. The claim does not require the mouse have a non-wild type phenotype, which is required for reasons cited above. Given the unpredictability in the art taken with the guidance provided in the specification, the cell in a) should be a mouse ES cell, the blastocyst in b) should be a mouse blastocyst, and the transgenic mouse produced should have a genome comprising a homozygous disruption in Kir5.1, wherein said mouse lacks functional Kir5.1.

Claims 10-12 are directed toward methods of screening compounds using a cell or mouse having a disruption in a Kir5.1 gene. Step (c) requires determining whether the expression or function of Kir5.1 is modulated but the mice and cells do not express Kir5.1. The specification does not teach how to determine Kir5.1 expression in mice having a disruption in Kir5.1. While the specification teaches transgenics expressing LacZ, the specification does not teach how to use such mice in an assay to determine whether a compound modulates Kir5.1. Without such a disclosure, the specification does not provide adequate guidance for one of skill to use the mouse disclosed to determine compounds that modulate Kir5.1 expression or function.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 10-12, 14, 15, 17, 18, 21, 24 and 25 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 10-11 are indefinite because the mice do not express Kir5.1; therefore, Kir5.1 expression cannot be tested as claimed.

Claim 14 is indefinite because the metes and bounds of what applicants consider "significant" expression cannot be determined.

Claims 17 and 18 are indefinite because "increased anxiety" and "stimulus processing disorder" do not further limit "increased acoustic startle response" in parent claim 16. If claims 17 and 18 do further limit the acoustic startle response or the function of the mouse, it cannot be determined how. The startle test is generic

numerous nervous, muscle and cognitive functions (pg 54, lines 20-30). The limitations do not further limit a characteristic of the mouse because all mice having increased acoustic startle response are considered to have increased anxiety or stimulus processing disorder as claimed.

Claim 21 does not further limit claim 20 because all mice having dwarfism have decreased body weight.

Claim 25 is indefinite because phenotypes "associated" with a disruption in Kir5.1 cannot be determined. While the mice having a disruption in Kir5.1 have dwarfism and increased response to the startle test, it cannot be determined if those phenotypes are "associated" with Kir5.1 in humans. It is unclear if mice having a disruption in a gene mapped to the distal region of mouse chromosome 11 (see Mouri pg 182, Fig. 1, and col. 2, "additional comments") are "associated" with a disruption in Kir5.1.

Claim 25 is indefinite because it does not recite how to determine whether an agent ameliorates a phenotype and neither does the specification. It is unclear what controls are required and how such a determination is made. It is also unclear why a mouse having a disruption in Kir5.1 is required because any mouse can be used to determine whether a compound increases body size, body weight, spleen weight or spleen weight:body weight ratio.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 3-9 and 14 and 24 are rejected under 35 U.S.C. 103(a) as being unpatentable over Signorini (1997, PNAS, Vol. 94, pg 923-927) in view of Mouri (Genomics, 1998, Vol. 54, pg 181-182).

Signorini taught making a transgenic mouse having a disruption in an inward rectifier protein (GIRK2/Kir3.2) (pg 924, col. 2, 2nd ¶). Signorini did not teach disrupting the Kir5.1 gene in the mice.

However, Mouri taught the nucleic acid sequence of the mouse Kir5.1 gene (GenBank Accession No: AB016197).

Thus, it would have been obvious to one of ordinary skill in the art at the time the invention was made to make a transgenic mouse having a disruption in an inward rectifier protein as taught by Signorini wherein the inward rectifier protein was Kir5.1 as taught by Mouri. One of ordinary skill in the art at the time the invention was made would have been motivated to disrupt the Kir5.1 gene instead of the Kir3.2 gene to determine the function of Kir5.1 in the brain *in vivo*.

Thus, Applicants' claimed invention, as a whole is prima facie obvious in the absence of evidence to the contrary.

Conclusion

No claim is allowed.

Inquiry concerning this communication or earlier communications from the examiner should be directed to Michael C. Wilson who can normally be reached on Monday through Friday from 9:00 am to 5:30 pm at (703) 305-0120.

Questions of a general nature relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-1235.

If attempts to reach the examiner, patent analyst or Group receptionist are unsuccessful, the examiner's supervisor, Deborah Reynolds, can be reached on (703) 305-4051.

The official fax number for this Group is (703) 872-9306.

Michael C. Wilson

MICHAEL WILSON
PRIMARY EXAMINER

Form PTO -1449

SHEET 1 OF 1

INFORMATION DISCLOSURE CITATION FORM PTO-1449	Atty. Docket No.: **R-902**	Serial No.: **10/005,202**
	Inventor: **ALLEN**	
	Filing Date: **December 4, 2001**	Group Art Unit: **1632**

U.S. PATENT DOCUMENTS

EXAMINER'S INITIALS	REF.	PATENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE

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FOREIGN PATENT DOCUMENTS

EXAMINER'S INITIALS	REF.	PATENT NO.	DATE	COUNTRY	CLASS	SUBCLASS	Translation Yes	Translation No

OTHER DOCUMENTS

EXAMINER'S INITIALS	REF.	(Including Author, Title, Date, Pertinent Pages, Etc.)
MW	AA	T. Mouri et al., "ASSIGNMENT OF MOUSE INWARDLY RECTIFYING POTASSIUM CHANNEL *Kcnj16* TO THE DISTAL REGION OF MOUSE CHROMOSOME 11," Genomics, Vol. 54, September 1998, pp. 181-182
MW	AB	J. Leaney et al., "THE ROLE OF MEMBERS OF THE PERTUSSIS TOXIN-SENSITIVE FAMILY OF G PROTEINS IN COUPLING RECEPTORS TO THE ACTIVATION OF THE G PROTEIN-GATED INWARDLY RECTIFYING POTASSIUM CHANNEL," Proc. Natl. Acad. Sci., Vol. 97, No. 10, May 9, 2000, pp. 5651-5656

EXAMINER: MW	DATE CONSIDERED: 10-17-03

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformity and not considered. Include copy of this form with next communication to applicant.

Notice of References Cited	Application/Control No. 10/005,202	Applicant(s)/Patent Under Reexamination ALLEN, KEITH D.	
	Examiner Michael C. Wilson	Art Unit 1632	Page 1 of 2

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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Signorini, 1997, PNAS, Vol. 94, pg 923-927
	V	Wagner (May 1995, Clin. and Experimental Hypertension, Vol. 17, pages 593-605
	W	Mullins (1996, J. Clin. Invest., Vol. 98, pages S37-S40
	X	Wall (1996, Theriogenology, Vol. 45, pg 57-68

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited	Application/Control No. 10/005,202	Applicant(s)/Patent Under Reexamination ALLEN, KEITH D.	
	Examiner Michael C. Wilson	Art Unit 1632	Page 2 of 2

U.S. PATENT DOCUMENTS

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*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
X	U	Mouri (Genomics, 1998, Vol. 54, pg 181-182
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 923–927, February 1997
Genetics

Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K^+ channel GIRK2

(GIRK1/embryonic stem cells/genetics/weaver/cerebellum)

STEFANO SIGNORINI*†, Y. JOYCE LIAO‡, STEPHEN A. DUNCAN*, LILY Y. JAN‡§, AND MARKUS STOFFEL*†¶

Laboratories of *Metabolic Diseases and †Molecular Cell Biology, Rockefeller University, New York, NY 10021; and ‡ Departments of Physiology and Biochemistry and §Howard Hughes Medical Institute, University of San Francisco, San Francisco, CA 94143-0724

Contributed by Lily Y. Jan, November 21, 1996

ABSTRACT **G protein-gated, inwardly rectifying K^+ channels (GIRK) are effectors of G protein-coupled receptors for neurotransmitters and hormones and may play an important role in the regulation of neuronal excitability. GIRK channels may be important in neurodevelopment, as suggested by the recent finding that a point mutation in the pore region of GIRK2 (G156S) is responsible for the weaver (*wv*) phenotype. The GIRK2 G156S gene gives rise to channels that exhibit a loss of K^+ selectivity and may also exert dominant-negative effects on $G_{\beta\gamma}$-activated K^+ currents. To investigate the physiological role of GIRK2, we generated mutant mice lacking GIRK2. Unlike *wv/wv* mutant mice, GIRK2 −/− mice are morphologically indistinguishable from wild-type mice, suggesting that the *wv* phenotype is likely due to abnormal GIRK2 function. Like *wv/wv* mice, GIRK2 −/− mice have much reduced GIRK1 expression in the brain. They also develop spontaneous seizures and are more susceptible to pharmacologically induced seizures using a γ-aminobutyric acid antagonist. Moreover, *wv*/− mice exhibit much milder cerebellar abnormalities than *wv/wv* mice, indicating a dosage effect of the GIRK2 G156S mutation. Our results indicate that the weaver phenotypes arise from a gain-of-function mutation of GIRK2 and that GIRK1 and GIRK2 are important mediators of neuronal excitability *in vivo*.**

G protein-gated, inwardly rectifying K^+ channels (GIRK) are regulated by neurotransmitters and hormones through G protein-coupled receptors (1–3). GIRK channels are believed to determine neuronal membrane excitability by selectively permitting the flux of K^+ ions near the resting membrane potential (4–7). The weaver mouse, a neurological mutant characterized by extensive cerebellar granule cell death during development (8–10), age-dependent dopaminergic neuronal loss in the substantia nigra (11, 12), male infertility (13), and spontaneous seizures (14), carries a G156S point mutation in the pore-forming region H5 of GIRK2 (15). This mutation leads to a loss of K^+ selectivity of homomeric GIRK2 channels and strongly reduces heteromeric GIRK1/GIRK2 channel function (16–18). Electrophysiological recordings from weaver and wild-type cerebellar granular cells have yielded conflicting reports, supporting either a loss of K^+ selectivity (16) or a loss of channel function (19). To study the physiological effects of GIRK2 *in vivo* and to address the question whether the phenotypic defects in the weaver mouse are due to gain-of-function effects such as the loss of K^+ selectivity or due to loss-of-function or dominant-negative effects on GIRK1/GIRK2 heteromultimeric channels, we have generated GIRK2-deficient mice and compared them to mice carrying one or two copies of the *wv* allele but no wild-type GIRK2 gene.

MATERIALS AND METHODS

Genomic Cloning and Construction of a Targeting Vector. Genomic clones containing the murine GIRK2 gene were isolated from a λFIX II murine 129/Sv genomic library (Stratagene) by screening the library using the full-length hamster GIRK2 cDNA as a probe (20). Two identical phage clones containing the entire murine GIRK2 gene were identified, and three exons containing the entire open reading frame were mapped. To generate the GIRK2 targeting vector pPNT-76, an ≈8-kb *Eco*RI fragment containing exon 1 and part of exon 2 was inserted into the targeting vector pPNT (21) such that its 3′ end was adjacent to the PGK promotor upstream of the neomycin gene. The 3′ end of the targeting construct was generated from the same GIRK2 genomic clone and contained a 0.47-kb *Bgl*II–*Xba*I fragment that was inserted into the exon 2 *Eco*RI/*Bgl*II deletion and included sequences from exon and intron 2 (Fig. 1*A*). The targeting vector was linearized by *Not*I and electroporated into R1 embryonic stem (ES) cells at 200 V and 800 mF. Stable colonies were grown under double selection in 350 μg/ml G418 and 0.2 mM gancyclovin in ES cell medium (22). By Southern blotting, 150 colonies were analyzed for homologous recombination. One clone (G2) was identified by the presence of a 1.9-kb *Bgl*II band and was microinjected into blastocysts to generate GIRK2-deficient mice (Fig. 1*B*).

Western Blot Analysis. Mouse brain membrane (50 μg), prepared as described (23, 24), was solubilized in 2% SDS/sample buffer (125 mM Tris, pH 6.8/20% glycerol/5% 2-mercaptoethanol) and loaded onto each lane. Western blots were probed with 1 μg/ml affinity-purified rabbit polyclonal antibodies against the N terminus of GIRK2 or GIRK1 or against the C terminus of IRK1 (23). Donkey anti-rabbit-horseradish peroxidase was used as secondary antibody at 1:5000 dilution. The blots were developed with enhanced chemiluminescence reagents (Amersham) and exposed to Hyperfilm-ECL (Amersham).

Immunohistochemistry. Mice were perfused intracardially with 4% formaldehyde and 0.1% glutaraldehyde in PBS (pH 7.4). Brains were dissected and postfixed overnight at 4°C. Fifty-micrometer vibratome sections were collected in 0.1 M Tris (pH 7.6); blocked with 2% H_2O_2; washed in 50 mM Tris, pH 7.5/100 mM NaCl/0.1% Triton X-100 (TBST); and then blocked in 4% normal goat serum and 3% BSA in TBST.

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0027-8424/97/94923-5$2.00/0
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Abbreviations: PTZ, pentylenetetrazol; ES cells, embryonic stem cells; RT, reverse transcriptase; TH, tyrosine hydroxylase.

¶To whom reprint requests should be addressed at: Laboratory of Metabolic Diseases, Rockefeller University, New York, NY 10021.

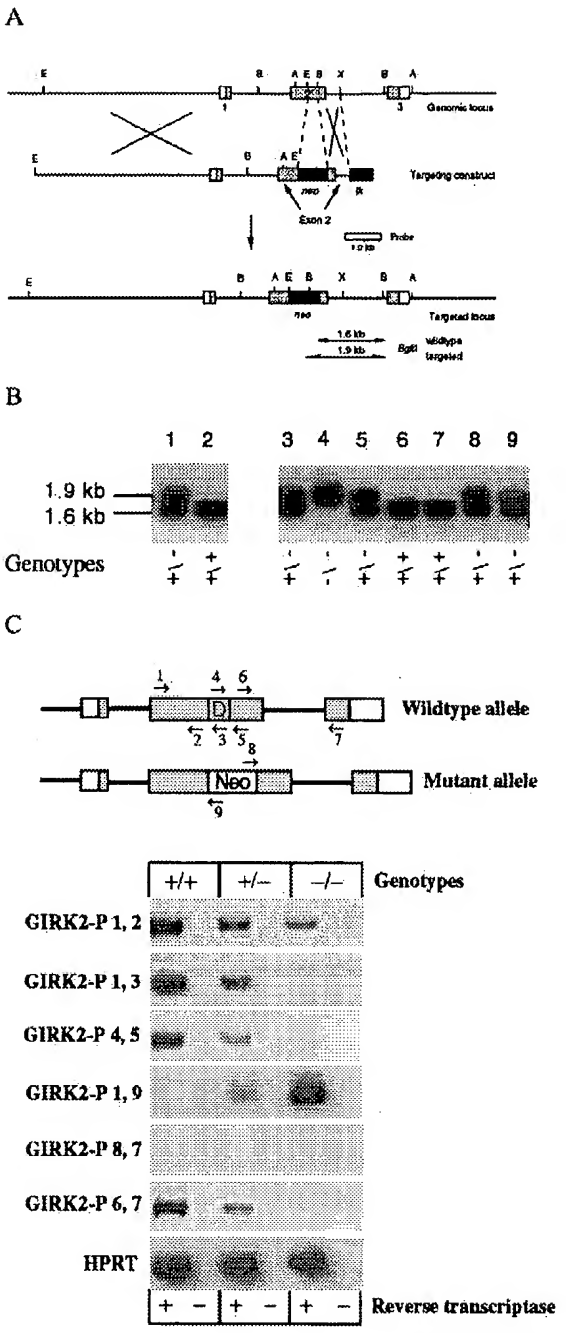

FIG. 1. (*A*) Targeted disruption of the mouse GIRK2 gene in ES cells and mice. The genomic structure and restriction map of the mouse GIRK2 gene locus and targeting vectors pPNT-76 used to disrupt the GIRK2 gene are shown. Shaded boxes represent coding sequences of the exons (boxes). The probe 3′ of the deletion is used for Southern blot analysis and shown as an open bar. B, *Bgl*II; A, *Acc*I; E, *Eco*RI; X, *Xba*I. (*B*) Southern analysis of transfected ES cells. Lanes: 1, targeted clone containing the targeted allele, as assessed by the presence of an additional 1.9-kb *Bgl*II fragment; 2: parental clone containing the normal 1.6-kb fragment; 3–9, genotypes from tail biopsies of GIRK2 +/+, +/−, and −/− mice. (*C*) Reverse transcriptase (RT)-PCR analysis from brain mRNA of GIRK2 +/+, +/−, and −/− mice. (*Upper*) GIRK2 wild-type and mutant alleles are shown with the position of oligonucleotides used as PCR primers. D, deleted region into which the pgk-neomycin resistance cassette was inserted. No primer-pair amplified products in the absence of reverse transcriptase. Each sample started with an equal amount of cDNAs. A 5′-truncated mRNA terminating in pgk-neomycin can be detected by PCR at reduced levels in +/− and −/− animals. The sequences of the oligonucleotide used are available upon request.

Rabbit antibodies were affinity-purified, and sections were incubated in 1–2 μg/ml primary antibody overnight (23, 24). Monoclonal antibodies against tyrosine hydroxylase (TH; Pel-Freez Biologicals) were used at 1:1000. Biotinylated donkey anti-rabbit or anti-mouse IgG Fab (The Jackson Laboratory) were used at 1:200, and sections were developed with the ABC kit (Vector Laboratories) and diaminobenzidine.

Induction of Seizures Using Pentylenetetrazole (PTZ). PTZ (Sigma) was dissolved in PBS and injected i.p. at a dose of 50 mg/kg in ≈0.1 ml. Animals were housed in a room with controlled light/dark cycle (12 hr light/12 hr dark) and temperature (23°C). All experiments were performed between 11 a.m. and 1 p.m. Animals were injected and observed without prior knowledge of their genotype. Each mouse was placed in a transparent cage and observed for 30 min after injection. All mice were littermates between 10 and 14 weeks of age and weighed ≥20 g.

RESULTS AND DISCUSSION

Generation of GIRK2 Null Mice. The GIRK2 gene was disrupted in ES cells by homologous recombination using a targeting vector in which exon 2 was disrupted and partially deleted by a pgk-neomycin resistance cassette (Fig. 1*A*). One ES cell clone that carried the targeted allele was used to generate chimeric male animals that passed the mutant allele to their offspring. GIRK2 +/− mice were indistinguishable from wild-type mice and were inbred to produce GIRK2 −/− mice (Fig. 1*B*). No normal GIRK2 mRNA could be detected in brains of adult GIRK2 −/− mice by RT-PCR analysis, but a truncated GIRK2 mRNA was present (Fig. 1*C*). No GIRK2 immunoreactivity was detectable using antibodies against either the N terminus or C terminus of GIRK2 (Figs. 2*A* and 3 *A* and *B*; data not shown). We conclude, therefore, that we have generated GIRK2 null mice.

Down-Regulation of GIRK1 Protein in GIRK2 Null Mice. GIRK2 −/− mice are born at the expected frequency and are viable. Given that GIRK2 and GIRK1 have partly overlapping temporal and spatial expression patterns and are known to form functional heteromultimers *in vitro* (16–18, 25–28) and *in vivo* (24), we examined the expression of GIRK1 and other related inward rectifier channels by using affinity-purified polyclonal antibodies against GIRK1, IRK1, and GIRK4 in Western blot and immunohistochemical studies of GIRK2 +/+, +/−, and −/− mice. Immunoblot analysis showed that GIRK1 levels were reduced in brain membranes of GIRK2 +/− mice and nearly undetectable in −/− mice, whereas IRK1 protein levels remained constant in mice of all three genotypes (Fig. 2*A*). RT-PCR analysis showed that GIRK1, GIRK4, and IRK1 mRNA were similar in all animals, suggesting that the down-regulation of GIRK1 in GIRK2 −/− mice occurred posttranscriptionally (Fig. 2*B*). Immunohistochemical analysis showed dramatic reduction of GIRK1 immunoreactivity in many brain regions in GIRK2 −/− mice, whereas IRK1 and GIRK4 immunoreactivities were normal in these GIRK2 mutants (Fig. 3 *A* and *B*; data not shown). The extent of reduction in GIRK1 varied with the brain regions; expression of GIRK1 in the cerebral cortex and hippocampus was virtually undetectable, whereas in the cerebellum, significant amounts of GIRK1 remained in the granule cell layer (Fig. 3 *A* and *B*). The reduction of GIRK1 protein levels throughout the brain suggests that a majority of GIRK1 proteins in the brain interact with GIRK2, and in the absence

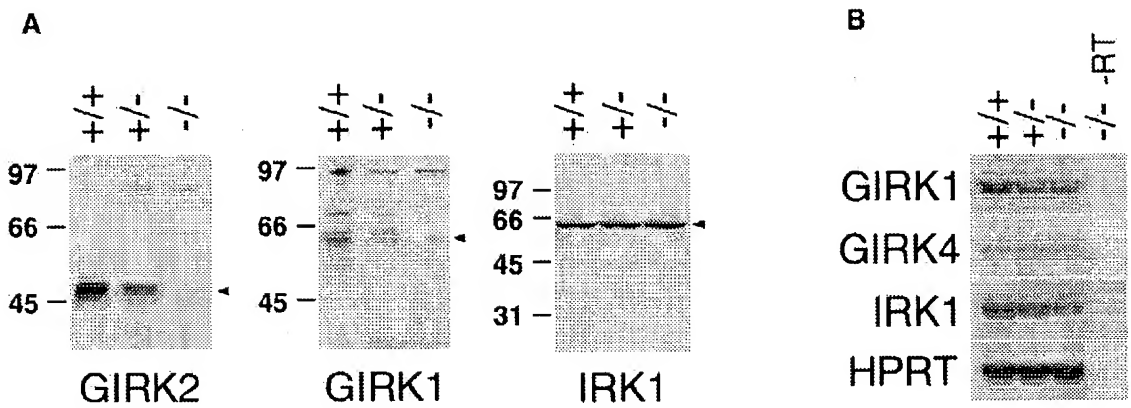

FIG. 2. (*A*) Western blots of membrane prepared from GIRK2 +/+, +/−, and −/− mouse brains show that both GIRK2 and GIRK1 protein levels are reduced in the GIRK2 knockout mice. (*B*) RT-PCR showing equal mRNA expression of GIRK1 in +/+, +/−, and −/− mouse brain. No product is amplified by any of the four pairs of primers in the absence of reverse transcriptase (−/− −RT; lane 4), confirming that all products were amplified from cDNA rather than contaminating genomic DNA. Hypoxanthine phosphoribosyltransferase (HPRT) primers amplify a comparable level of product in all samples, indicating that the same amount of template is present. Amplified products by GIRK1, GIRK4, IRK1, and HPRT primers are of expected sizes.

of GIRK2, there is a concurrent loss of GIRK1 subunits that normally form heteromultimers with GIRK2.

Differences Between the GIRK2 Null Phenotypes and the Weaver Phenotypes. The GIRK2 −/− and *wv/wv* mice showed striking differences. Visual inspection and histological examination of the brain and other organs of GIRK2 −/− animals revealed no anomalies. GIRK2 −/− mice exhibited normal cerebellar morphology except for the reduced GIRK1 and GIRK2 protein expression (Fig. 3 *B* and *D*). Midbrain dopaminergic neurons and their dendrites also appeared normal despite the absence of GIRK2 protein (Fig. 3*C*). While male *wv/wv* mice are infertile, male GIRK2 −/− mice are fertile; superovulated CD-1 mice mated with either GIRK2 −/− males or their wild-type littermates produced a comparable number of fertilized eggs. The apparent normal phenotype in GIRK2 −/− mice provides strong evidence that loss of homomeric GIRK2 channel and/or heteromeric GIRK1/GIRK2 channel function is not the primary cause of the weaver phenotype.

The Weaver Gene Dosage Effect. When GIRK2 −/− mice were compared with mice carrying one or two copies of the *wv* allele (GIRK2 *wv*/− and/or *wv/wv*), we found that both −/− and *wv*/− mice exhibit normal locomotive behavior, unlike the *wv/wv* mice. In +/+, −/−, and *wv*/− animals, there was no obvious loss of TH-positive neurons or dendrites in the substantia nigra pars compacta or in the ventral tegmental area, whereas substantial cell loss was evident in the substantia nigra pars compacta of the *wv/wv* midbrain (Fig. 3*C*). In the substantia nigra pars compacta and ventral tegmental area of *wv*/− mice, GIRK2 immunoreactivity was present but of lower intensity. In contrast to the heterozygous GIRK2 +/− mice, most of the GIRK2 immunoreactivity in the *wv*/− mice was found in the cell bodies of the dopaminergic neurons; the GIRK2 immunoreactivity in the dendrites was much reduced (Fig. 3*C*). The size and gross morphology of the cerebellum of *wv*/− animals are not significantly different from that of the wild-type animals. Histologically, the *wv*/− cerebellum appeared more similar to that of the *wv*/+ (8, 9) than the cerebellum of +/+ or *wv/wv* mice. The *wv*/− granule cell layer often appeared thinner. The Purkinje cell layer was disorganized in various locations, and some of the Purkinje cells were found deep in the granule cell layer (Fig. 3*D*). The similarity between *wv*/− and *wv*/+ cerebella and the difference between *wv*/− and *wv/wv* cerebella suggest that cerebellar development is sensitive to the dosage of the GIRK2 G156S mutant gene.

Seizure Activities of GIRK2 Null Mice. The GIRK2 −/− mice exhibited sporadic seizures characterized by jerking of head and body, vocalization, and infrequently progression to a tonic-clonic seizure. Typically, the episodes lasted for 30 sec and were followed by complete physical inactivity. All witnessed seizures occurred when some kind of stress was exerted on the animal (changing cages, setting up matings), and the behavior of mice returned to normal after the seizure. Seizures were never observed before weaning and seemed to occur at equal frequencies in young and old mutant mice. Pharmacological challenge with the convulsant agent PTZ (29), a γ-aminobutyric acid antagonist, revealed that GIRK2 −/− mice were hyperexcitable when challenged with a single injection of PTZ (50 mg/kg). At this dose, 70% of GIRK2 −/− mice but only 25% of heterozygous or wild-type littermates developed severe stage 3 tonic-clonic seizures frequently associated with death ($P < 0.004$, Mann–Wilcoxon rank sum test). The severity of seizure, in the range from 0 to 3, was shifted toward increased severity in GIRK2 −/− mice as compared with +/− and +/+ controls. No statistically significant difference was seen between heterozygous and wild-type mice (Fig. 4*A*). The time taken to develop seizure activities was significantly shorter in GIRK2 −/− mice compared with +/− and +/+ animals ($P < 0.002$, unpaired *t*-test; Fig. 4*B*). Seizure activity has previously been noted in weaver mice and might be due to altered or reduced G protein-activated K^+ channel function (14). Our observation that GIRK1/GIRK2-deficient mice are susceptible to spontaneous and pharmacologically induced seizures was consistent with numerous studies demonstrating that agonists of G protein-coupled receptors, such as receptors for opioid peptides, somatostatin, and dopamine, can have significant effects on seizure thresholds in several different experimental seizure model systems (30).

In conclusion, we show that GIRK2-deficient mice have greatly reduced GIRK1 protein levels in the brain, suggesting that the majority of GIRK1 proteins in the brain associate with GIRK2. Phenotypic characteristics of GIRK2 −/−, *wv*/−, *wv*/+, and *wv/wv* mice suggest that gain-of-function and gene dosage mechanisms are responsible for the developmental defects in weaver mutants. Moreover, loss of GIRK2 function results in sporadic seizures and increased susceptibility to a convulsant agent, implicating GIRK1 and GIRK2 in the control of neural excitability *in vivo*.

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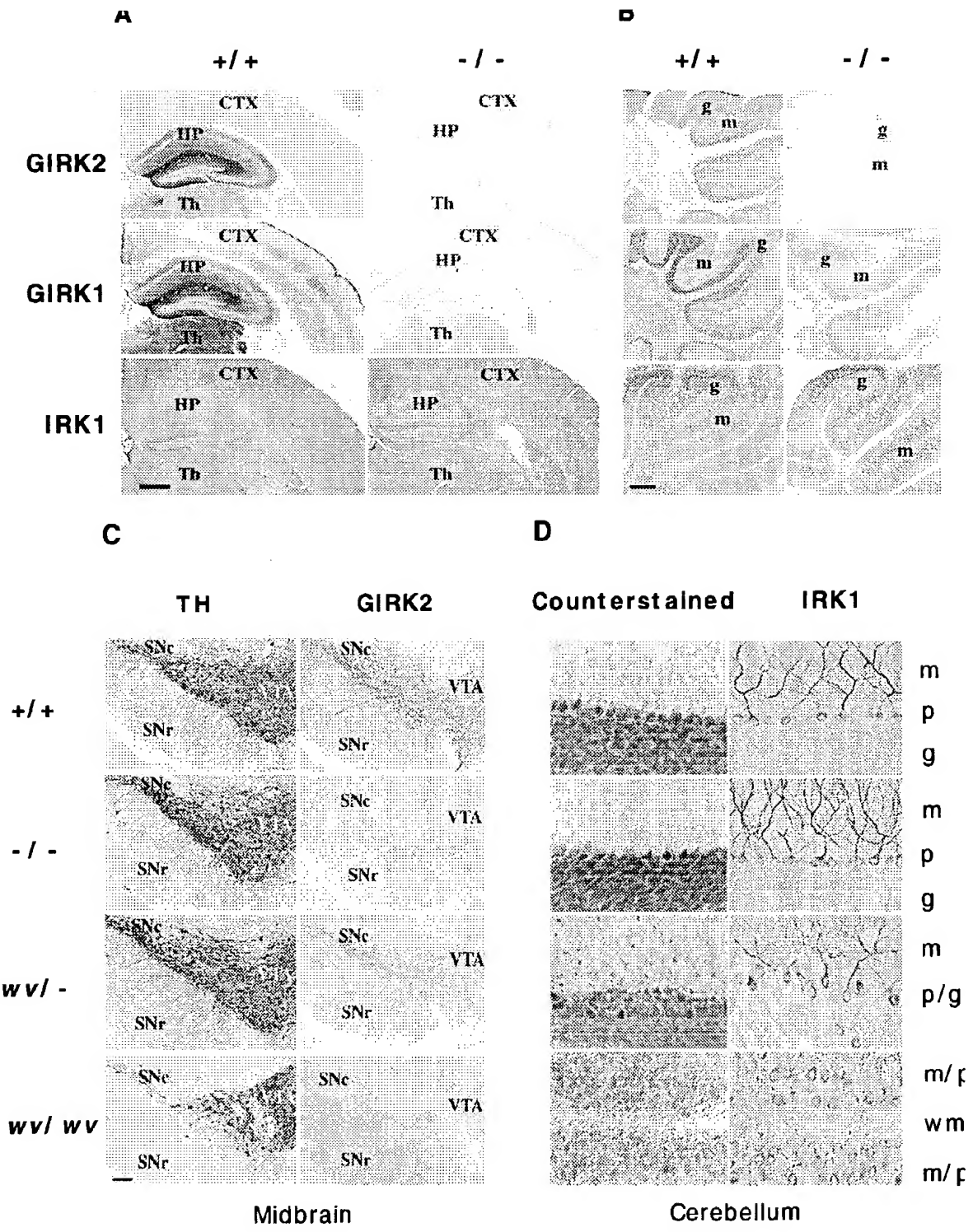

FIG. 3. (*A*) Coronal sections of brain from GIRK2 +/+ and −/− mouse brains stained with antibodies against the N terminus of GIRK2, the N terminus of GIRK1, and the C terminus of IRK1 show that there is no detectable GIRK2 and dramatically reduced GIRK1 immunoreactivity in the GIRK2 −/− mice. The IRK1 staining patterns are the same for these mice. CTX, cerebral cortex; HP, hippocampus; Th, thalamus. (Bar = 10 mm.) (*B*) Sagittal views of cerebella from GIRK2 +/+ and −/− mice stained with the antibodies described in *A*. Although there is no detectable GIRK2 protein in the −/− mice, there is still significant GIRK1 staining, whereas the level of IRK1 expression appears to be the same. g, granule cell layer; m, molecular layer. (Bar = 20 mm.) (*C*) Coronal sections of ventral midbrain from +/+, −/−, *wv*/−, and *wv*/*wv* mice are stained with antibodies against TH and the N terminus of GIRK2. The TH staining of +/+, −/−, and *wv*/− midbrain appears similar, whereas there are fewer TH-positive neurons in the substantia nigra pars compacta (SNc) of the *wv*/*wv* midbrain. GIRK2 immunoreactivity is absent in the −/− midbrain but can still be found in the *wv*/− and *wv*/*wv* mice, although the dendritic staining is dramatically reduced. SNr, substantia nigra pars reticulata;

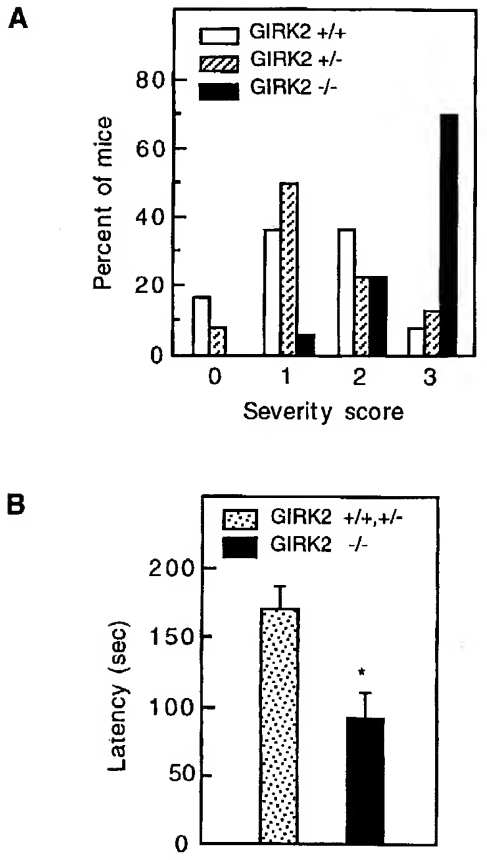

FIG. 4. Susceptibility of GIRK2-deficient mice to PTZ-induced seizures. (*A*) Response of mice receiving one injection of 50 mg/kg PTZ i.p. (0, no response; 1, isolated twitches; 2, tonic-clonic convulsions; 3, tonic extension and/or death). GIRK2 −/− mice (n = 16) tend to progress to more severe stages than +/+ or +/− mice (n = 13 and 12, respectively; $P < 0.004$, Mann–Whitney U–Wilcoxon rank sum test). No statistically significant difference was observed between +/+ and +/− animals. (*B*) Seizure latency. The PTZ seizure latency was defined as the time elapsed from PTZ injection to the first obvious sign of tonic-clonic convulsion or tonic extension. The latency to seizures was shorter for the −/− mice (*, $P < 0.002$, unpaired test).

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VTA, ventral tegmental area. (Bar = 1 mm.) (*D*) High magnification views of parasagittal cerebellar sections from +/+, −/−, *wv*/−, and *wv*/*wv* mice. The sections are counterstained with toluidine blue or stained with antibody against the C terminus of IRK1, which stains the cell body and dendrites of the Purkinje cells as well as the dendrites in the granule cell layer. The *wv*/− cerebellum is mostly wild-type in appearance except for regions with a Purkinje cell layer more disorganized and broader than that in GIRK2 +/+ and −/− mice. Some Purkinje cells and their dendrites can be found in the granule cell layer of *wv*/− mice. In the *wv*/*wv* cerebellum, there is no granule cell layer, and the Purkinje cells with disorganized dendrites are scattered throughout the cerebellum. m, molecular layer; p, Purkinje cell layer; g, granule cell layer; p/g, cell layer where both Purkinje cells and granule cells are found; m/p, cell layer where molecular layer and Purkinje cell layers collapse into one in the absence of granule cell layer; wm, white matter. (Bar = 0.2 mm.)

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REVIEW

TRANSGENIC ANIMALS AS MODELS FOR HUMAN DISEASE

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Keywords: Transgenic animals, human disease, genetics, cardiovascular disease, oncology, immunology, toxicology, Alzheimer's disease, embryo development

Abstract

Since its first description in 1981 (1), transgenic technology has greatly influenced the focus and direction pace of biomedical research. Introduction of foreign DNA into the genome of animals by microinjection into fertilized oocytes is now used in almost every field of research spanning from oncology, immunology and neurology to cardiovascular medicine. The ability

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to integrate genes in the germline and their successful expression in the host provides an opportunity to study the role of a certain gene in the initation and propagation of disease. Transgenic methodology serves as the link between molecular biology, introducing *in vitro* a defined genetic modification and whole animal physiology, with the resulting *in vivo* alteration of body function. This potential has been exploited to study the pathophysiological role of human genes. Transgenic animals have been used to study aspects of tumor development, immune regulation, cardiovascular development and atherosclerosis. These studies have provided new insights into the genetic origin of certain diseases and have improved our understanding of pathological processes on the cellular level. As a future goal, these studies may also serve the development of new diagnostic tools or novel therapeutic strategies such as gene therapy.

Introduction

Our understanding of body function in health and disease has been advanced in the past primarily by the use of animal models for human disease. However, to establish a relationship between the regulation of a certain gene and a complex disease process has been difficult. The expression of a foreign gene creates a defined genetic defect, which allows to closely correlate the effect of this gene to a physiological trait.

Studies on the regulation of gene expression and gene function in humans are strongly limited due to ethical reasons. Expression of human transgenes in animals therefore is an elegant way to obviate these difficulties. Experiments using transgenic animals can be divided into four categories: 1) Studies on gene regulation: which include the expression of only the regulatory elements of a gene such as the promoter region in transgenic animals. The promoter region of a gene is connected to a reporter gene which is easilly detectable. Such studies using the promoter region are of use in the analysis the regulatory elements for tissue-specific expression and identification of cis-acting factors controlling gene transcription. 2) Investigation of the function of a gene product: Here, the gene of interest is under control of either the natural homologous promoter or a heterologous one which directs expression to specific cells and tissues. Additionally, mutants of the gene of interest may be introduced for

analysis of specific biochemical properties. 3) *In vivo* immortalization of cells: this is accomplished e.g. by fusion of a gene promoter region to a SV 40 large T antigen. The immortalized cell lines are then isolated for *in vitro* analysis. Finally 4) expression of proteins in mammary gland tissue in order to obtain large quantities of secretory protein within the milk.

Transgenic technology was originally developed in the mouse and, therefore, most transgenic studies are performed in this species. The mouse model provides serveral advantages such as comparatively numerous offspring with short generation times and well-known genetics. For specifc research questions, however, other species have been used such as rat, sheep, rabbit, goat and zebrafish (2-5). In cardiovascular research rats have been the animals of choice to study cardiovascular function. They are suitable for pharmacological tests and have provided animal models for hypertension, cardiac and renal disease.

The most widely used technique applied for transgenic production is micromanipulation of fertilised oocytes from superovulated donor animals and microinjection of DNA into the pronucleus (1,6). The DNA-injected eggs are reimplanted into pseudopregnant foster mothers and the offspring are then analysed for the presence of the foreign DNA in the genome. This technique is basically common to all animal species where this technology has been applied. Time schedule, hormone treatment and operating procedure, however, require adjustment to the respective species. Whereas microinjection aims at implantation of foreign genomic material into the germline, different techniques have been developed to insert DNA into *somatic* cells (7-10). This implies transgenesis in cells which are not part of the germline and, therefore, a genetic alteration can not be propagated to the offspring. These techniques often aim at gene therapeutic approaches where a defective gene can be replaced in function at some time in the ontogeny.

Three methodological approaches have been used to generate transgenic animals: i) injection of DNA into the pronucleus of fertilized oocytes as described above. ii) homologous recombination in embryonic stem cells of mice (11) and iii) retroviral infection of preimplantative blastocysts (12). At present, research efforts are focused on the first two techniques which represent two sides of a coin. The "knock out" approach achieved with homologous recombination yields a "loss-of-function" study, in contrast to the "gain-of-

function" approach which is used in microinjection causing overexpression of a gene. "Loss-of-function" is a tool to analyse the function of a gene by functional interruption and is achieved by inserting a disruptive sequence *in vitro*. The endogenous gene is replaced with the mutated gene by homologous recombination in the stem cell. Despite the precision of this method, functional conclusions are not easily drawn from such experiments. For example, gene "knock-outs", especially of transcription factors, have often demonstrated no apparent phenotype due to the functional redundance of cell and gene regulatory systems . Other gene disruptions have precluded extensive analysis due to embryonic lethality. Studies using partial "loss-of-function"-mutants or double knock outs are under way to obviate these difficulties (13). Thus far, embryonic stem cell technology has only be successful in mice, however, the need to generate rat embryonic stem cells has been recognised for physiological and pharmacological investigations.

Homologous recombination allows replacement of a the native gene by a mutant which can be analysed in the natural chromosomal environment, which affects gene expression and regulation. In contrast, transgenesis by micorinjection occurs by random insertion into the genome without control of copy numbers of DNA integrated. To achieve a reproducible gene integration, locus control regions or matrix attachment regions have been used as control elements to direct transgene integration (14-17). Another successful strategy has been not to inject the transgene alone but rather large DNA constructs such as yeast artifical chromosomes (18-19), in order to control the "environment" of the transgene. These techniques will in the future redefine the transgenic methodology and possibly other newly developed strategies for somatic transgenesis. Progress in this field can not be separated from gene therapeutic approaches, where a foreign gene will be transfered into somatic cells. A vast number of different *in vitro* and *in vivo* strategies exists for gene transfer. *In vitro* strategies use host cells that are isolated from the body, grown , stably transfected with a transgene and then reimplanted (10). *In vivo* studies directly apply DNA to the host either by direct injection into the tissue of interest, by liposomal transfection, by retroviral or adenoviral infection (8,9) or by receptor-mediated uptake e.g. by via the transferrin receptor. A detailed description of these technologies, however, is beyond the scope of this article.

Transgenic animals in cardiovascular disease

The cardiovascular system has been the focus of interest for transgenic research due to the high cardiovascular morbidity and mortality in industrialized societies. Transgenic animals have been generated for almost every aspect of cardiovascular research from hypertension to formation of myocardial tumors. The candidate gene approach has been used to study the effects of gene products of hormones which are known to be involved in blood pressure regulation and which are supposed to play a role in the pathogenesis of hypertension. Other risk factors of cardiovascular disease such as atherosclerosis or hemostatic mechanisms have been investigated by transgenic techniques.

Candidate gene approach/neurohormonal studies

The regulation of cardiovascular function is complex and depends on many factors which interact in a defined spatial and temporal pattern. It is therefore difficult to assign a particular phenotype or functional parameter to a certain gene. Transgenic introduction of a gene into an organism does allow to define the contribution of a certain gene to the physiology or pathophysiology of cardiovascular function. Due to the multitide of hormones, regulatory peptides, cell signalling pathways etc., research has focused on the role of candidate genes. These are genes, which are known to be involved in cardiovascular regulation and, therefore, likely to play a role in dysfunotion of the heart or the vascular wall as in hypertension. Since the expression of the transgene in animals is the only difference to transgene-negative control animals, a change in cardiovascular function can be correlated to the presence of the transgene.

The precursor of *arginine vasopressin*, preproarginine vasopressin, which is under control of the metallothioncin promoter has been expressed in transgenic mice resulting in chronically elevated levels of vasopressin in the plasma (20). Increased levels of vasopressin were present in the plasma elevating serum osmolality to levels corresponding to mild nephrogenic diabetes insipidus. *Atrial natriuretic peptide* which is known to reduce blood pressure and to induce a

marked natriuresis has been expressed in mice to study the effects of chronically elevatd ANP levels on cardiovascular function. Use of the heterologous mouse promoter transthyretin resulted in a ten-fold elevation of immunoreactive plasma ANP and significantly lowered blood pressure without altering plasma electrolyte balance (21).

Transgenic animals have also been generated in hypertension research. Besides the known influence of environmental factors on the development of high blood pressure, hypertension has a strong genetic background (22,23). Therefore, candidate genes of hypertension such as the components of the *renin-angiotensin system* have been studied in detail. This system is a major regulator of blood pressure and of sodium- and volume homeostasis. Renin genes of different species, as well as its substrate angiotensinogen, have been introduced into transgenic mice (24-27). Transgenic mice expressing both the rat or human renin and angiotensinogen gene developed elevated blood pressure levels (27,28). However, rats, as opposed to mice, have attracted much interest in the field of research, since they are more suitable for hemodynamic, pharmacological and functional studies. Rats with hereditary hypertension, such as spontaneously hypertensive rats, have been used as a model for primary human hypertension. Expression of the mouse renin-2-gene in transgenic rats has led to fulminant hypertension with values in the range of 220 mmHg systolic in heterozygous animals (3). Unexpectedly, despite the presence of an additional renin gene, these rats exhibit a low plasma renin activity, corresponding to low renin hypertension syndromes in humans. Transgenic rats with the human renin and angiotensinogen gene have also been generated which maintain the species-specificity of the human renin-substrate reaction (4).

Cardiovascular aspects can also be demonstrated in transgenic mice overexpressing *growth hormone* (29,30). Excess growth hormone in humans causes acromegaly and gigantism. Patients suffering from this disease frequently develop hypertension, although growth hormone by itself is not hypertensinogenic. Overexpression of a metallothionein-fusion gene in mice did not significantly raise blood pressure, but the vascular wall-to-lumen ratio was significantly altered in mesenteric arteries. The increase in wall thickness in these arteries may elevate peripheral resistance and thus contribute to the hypertensive blood pressure levels in acromegalic patients. However, the multifocal expression of growth hormone has also a

number of other effects resulting in progressive glomerulosclerosis after induction of mesangial cell growth. (30)

In addition to hypertension, other risk factors of cardiovascular disease such as atherosclerosis have been investigated: Lipoproteins are the macromolecular transporters of non-polar lipids. The major high density lipoprotein (HDL) associated apolipoprotein is *apolipoprotein AI.* Plasma HDL concentrations as well as apoAI levels have been shown to be inversely correlated to the development of premature coronary heart disease. As its major apolipoprotein constituent, apoAI plays a central role in HDL assembly. The human apoAI gene was transferred into the atherosclerosis susceptible inbred mouse strain C57BL76. This transgene lead to a 2-fold increase in apoAI and HDL. Similarly, apolipoprotein E was expressed in transgenic mic (32). Although these mice were fed a high fat diet, they were markedly protected from atherosclerotic plaques. In other experiments, high levels of *the low density lipoprotein (LDL) receptor* was expressed using a receptor cDNA under the control of a metallothionein promoter. These mice cleared LDL from blood 8-times faster than normally. The transfer of LDL receptors to patients with known genetic LDL receptor defects as in familial hypercholesterolemia may be an approach do replace defective receptor function (33).

Transgenic animals in pulmonary disease

Most animal models which are used to mimic human disease are based on lesions which are applied to the adult animal and then the course of disease or the effect of treatment is analysed over time. Whereas animal models applicable to chronic and degenerative disease processes is less frequent developed. New animal models for chronic human disease may be generated by transgenic animals either by overexpression and excess function of a particular gene or by disruption of a functional gene. The success of these approaches depends on the extent to which a singular gene is indeed responsible for a disease. The investigation of *cystic fibrosis* the most common autosomal inherited disease, is a paradigm for these problems. The defective gene in cystic fibrosis patients has been identified as *Cftr*, which encodes an ion channel at the cell membrane. By homologous recombination, several groups succeeded to

disrupt the Cftr gene (34-36). All animal lines developed symptoms of cystic fibrosis. Although all these experiments created null mutations, the time course and the pattern of tissue involvement differed between the lines. These null mutation mice may be used for further studies on the *Cftr* gene, by introducing mutations of the *Cftr* gene via the transgenic approach. Comparison of the different clinically important mutations compromising the *Cftr* gene may clarify the tissue specific pattern of tissue involvement. Beyond that, these mice are excellent models to develop gene therapeutic strategies. Somatic transgenesis by liposomal transfection led to expression of a functional *Cftr* gene in the lung of these transgenic mice with the disrupted ion channel. (7). Gene therapeutical trials are now underway in humans with cystic fibrosis to apply the an intact *Cftr* gene supplementing the defective non-functional *Cftr* gene.

Transgenic animals in neurodegenerative disorders

As for chronic lung disease, transgenic methodology is being used to generate animal models which represent important aspects of human neurodegenerative disorders. The focus of interest has been *Alzheimer's disease*, where beta-amyloid, derived from the amyloid precursor protein, is chronically deposited in senile plaques and along vessel walls. In some forms of familial Alzheimer's disease, mutations of the amyloid precursor protein have been identified to be responsible for the development of the disease. Expression of the amyloid precursor protein in transgenic mice has not led to a distinctive Alzheimer's disease phenotype in the brain of these animals, however, a carboxyterminal fragment of the amyloid protein precursor causes neurodegeneration *in vivo* (37). Whether this closely correlates to Alzheimer's disease in humans is not clear and further studies aimed at overexpression of other components of the senile plaques such as the microtubule-associated protein *tau* are under way to generate an animal model for the human disease.

Transgenic animals in oncology or immunology

In these fields, most of the research employing transgenic technology is focused on fundamental studies rather than clinical disease. Several studies are being performed to

clarify the regulation of oncogenes. In particular, how they are activated and by which pathways these oncogenes exert their carcinogenic potential. Transgenic animals have been used as specific research tools to study the different steps in tumor development leading to a better understanding of the tumor-host interaction, tumor growth and angiogenesis and metastatic seeding. The analysis of these processes may lead to new therapeutic strategies, which could interrupt tumor development at the different stages. Similarly, the understanding of the immune system, the cytokine network and the host versus graft reaction in transplantation have been enhanced by transgenic approaches (38). The interaction of the cytokine network with antibody formation and B cell activity has been studied by "knock out" experiments. Gene targeting was used to disrupt genes involved in B and T cell differentiation (38,39). The role of cytokines and of differentiation factors in autoimmune and inflammatory disease has been investigated in several transgenic models . Overexpression of *human tumour necrosis factor alpha* in transgenic mice led to the development of chronic arthritis and systemic tumor necrosis factor-mediated disease (40). As a sign of chronic arthritis, accumulation of polymorphnuclear cells, synovial thickening and finally synovial hyperplasia and pannus, eroding the cartilage was observed. Similarly "knock out" of *transforming factor* β led to chronic and diffuse inflammation (41). How these experiments truely represent human syndromes of chronic inflammation remains to be elucidated, but they allow the investigation of the regulation of inflammatory processes.

Concluding remarks

Transgenic methodology has so rapidly developed that inclusion of all relevant data and publications is beyond the scope of this manuscript. However, it has been shown that transgenic methodology has spread into every field of biomedical research. Transgenic animals are being used to create new models for human disease, i) by generation of animal models which resemble the human condition as close as possible and ii) by in-detail analysis of the pathogenesis of human diseases under *in vivo* conditions. The further refinement of this technology from simple injection of foreign DNA into an oocyte to expression of large chromosomal regions and to the technique of embryonic stem cell manipulation underscores the fact that this technology is still in development.

Transgenic methodology, however, can not be considered in isolation: It is rather part of a major advancement gene technology has provided, extending into the fields of cell biology and molecular genetics. For examples, novel strategies in molecular genetics allow to identify the causative genes for disease and pathophysiological processes. In return. these genes can then be tested in transgenic animals on the functional and cellular level. In the future, these technologies can act in concert to develop new therapeutic strategies for human diseases and are therefore of interest for clinical applications. The methodological expansion, however, requires collaborative efforts of scientists and transfer of know-how between specialised laboratories more than ever.

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Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Transgenesis in the Rat and Larger Mammals

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Advances in biotechnology over the last ten years have made it possible for the researcher to alter gene expression in vivo in many diverse ways (1). With the establishment of embryonic stem (ES)[1] cell technology (2), more subtle and precise alterations can now be achieved than were previously possible using microinjection techniques. However, to date germline transmission has only been achieved with mouse ES cells, and microinjection continues to be the method most widely used for other species. While the mouse has a number of advantages, not least the depth of our knowledge of its genetics, other species are being increasingly used for transgenic studies due to their greater suitability for addressing specific questions. We will briefly review the application of transgenic technology to nonmurine species as it stands at present, with particular emphasis on developments appertaining to biomedical research.

Transgenesis by pronuclear injection

A number of significant limitations regarding the application of pronuclear injection to nonmurine animals have been identified (3), not least being the time and cost. Such limitations are due to longer gestation and generation times, reduced litter sizes, and higher maintenance costs. Further consideration must be given to the large numbers of fertilized eggs (and hence donor animals) required for microinjection, the high cost of carrying nontransgenic offspring to term, and the relatively low efficiency of gene integration. Such limitations are particularly severe for the production of bovine transgenics and, as a consequence, more significant departures from the standard procedures used for the mouse have been adopted for this species (4). For example, the use of in vitro embryo production in combination with gene transfer technology has played a large role in the development of transgenic cattle. The development of microinjected embryos through to the morula/blastocyst stage in recipient rabbits or sheep, enables sexing, transgene screening, and cloning to take place before reintroduction into the natural host, providing that such screening methods are robust and reliable.

The major problem regarding pronuclear microinjection is that the exogenous DNA integrates randomly into chromosomal DNA. Position effects, where the transgene is influenced by its site of integration in the host chromosome (5), can have major consequences on the expression of the transgene, including loss of cell specificity, inappropriately high copy number–independent expression and complete silencing of the transgene. This is of greater concern in nonmurine transgenesis where the investment is higher. Position-independent, copy number–related expression can be achieved using sequences such as the locus control regions identified upstream of the β-globin gene cluster and downstream of the CD2 gene (6, 7), the A elements which flank the chicken lysozyme gene (8), and matrix attachment regions (9). Such elements have been shown to function across species barriers, and their incorporation into gene constructs can overcome position effects and improve expression of heterologous genes within specific cell types (5). In many cases, simply including large amounts of flanking sequences may be sufficient to overcome position effects and direct expression to specific tissues. To this end, the development and use of P1 (10), bacterial artificial chromosome (BAC) (11) and yeast artificial chromosome (YAC) vectors (12) for cloning of large segments of DNA, should greatly improve the chances of including important regulatory elements, including those involved in chromatin structure, within the transgene construct.

Embryonic stem cell technology

With the development of ES cell technology in the mouse (2), genetic manipulations can be performed in cell culture using appropriate selection strategies to permit the directed integration of the transgene to a specific region of the chromosome via homologous recombination. With the advent of homologous recombination, the researcher is able to insertionally inactivate, replace, or introduce subtle alterations to the endogenous gene of interest. Once the intended genetic change has been verified, the appropriate ES cells are introduced into blastocysts by microinjection, and, during subsequent gestation, may contribute to the developing embryo. If such a contribution is made, then by definition the resulting animal would be chimeric, being derived in part from the ES cells originating in culture. Assuming that the chimerism extends to the germline, then an appropriate breeding strategy will lead to the recovery of nonchimeric heterozygotes and, if viable, mice which are homozygous for the genetic change.

Most attempts to isolate and culture inner cell mass (ICM) cells from other species are based on the methods used for the

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1. *Abbreviations used in this paper:* DAF, decay accelerating factor; ES, embryonic stem; HAR, hyperacute rejection; ICM, inner cell mass.

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mouse. ES cells are maintained in culture in the presence of mouse-derived differentiation-inhibiting agents, provided either as a media supplement or through cocultivation in the presence of feeder cells. It has been suggested that these mouse-derived agents do not adequately prevent differentiation of stem cells in species other than the mouse, and pluripotent rat ES cells, capable of producing chimeras, were found to grow best on primary rat embryonic fibroblasts as the feeder layer (13). Freshly isolated cells from ICMs have been injected into blastocysts to produce chimeric offspring in both sheep and cattle (14), and their totipotency at this stage is further demonstrated by their ability to produce offspring after transfer into enucleated oocytes (15). Such nuclear transfer techniques are potentially very useful for the production of clonal offspring and would avoid the initial chimeric generation necessitated by the injection of ES cells into blastocysts. Recently, bovine-specific culture methods have shown promise with cells of up to 27 d of age maintaining their ability to direct normal calf development following nuclear transfer (16). However, at the present time the reliable generation of bovine ES cell lines requires the pooling of ICMs from several blastocysts and further efforts are required to enable the long-term culture of clonal bovine ES cells. Although to date chimeric animals have been generated from several species including the pig (17), in no species other than the mouse has germline transmission of an ES cell been successfully demonstrated. This remains a major goal for the future and may well require the use of novel strategies which depart widely from the traditional methods used in the mouse.

Nonmurine species in biomedical research

Selected physiological questions may be more conveniently modelled in the rat or in larger species. Not only can physical size be an advantage for biochemical sampling and physiological analyses, but certain genes may provide useful information when introduced into, for example, the rat genome when parallel experiments in the mouse would be ineffective. Examples include the modulation of blood pressure by the mouse *Ren-2* gene (18) and the modeling of inflammatory disease (19). In both cases, but for different reasons, no phenotype was observed in the respective transgenic mice, highlighting one of the advantages of having alternative species for understanding physiological mechanisms and the etiology of disease. More recently, a number of transgenic experiments have been undertaken to investigate lipoprotein metabolism. The human apolipoprotein A-1 gene was successfully expressed in the rat (20), resulting in increased serum HDL cholesterol concentrations, and attempts to therapeutically lower apo B100, and hence LDL and lipoprotein(a) concentrations, in the rabbit were successful (21) but resulted in complications. Although the targeted expression of the apo B–editing protein in the liver of the transgenic rabbits resulted in reduced LDL and lipoprotein(a) concentrations as intended, many of the animals developed liver dysplasia, suggesting that high level expression of the editing protein had unforeseen and detrimental side effects, possibly via the editing of other important mRNAs. The rabbit has also been used in HIV-1 research, with the development of a line expressing the human CD4 protein on T lymphocytes (22). Susceptibility to HIV infection was demonstrated, and although the rabbits are less sensitive to infection than humans, they may represent an inexpensive alternative to primates for many studies.

Gene transfer in farm animals was initially aimed towards improving production efficiency, carcass quality (23), and disease resistance of livestock. However, it has been suggested that the simple over-expression of hormones such as growth hormone may have unacceptable side effects. Recently some elegant studies of growth using transgenic rats have been performed and are likely to yield valuable information on the biochemistry and physiology of growth (24, 25). A more successful application of transgenesis in farm animals has been the production of biomedically important proteins. The two most popular methods have been to direct expression to hematopoietic cells or to the lactating mammary gland. In the former case, transgenic swine expressing high levels of human hemoglobin were generated using the locus control region from the β-globin gene cluster to overcome positional effects and direct expression to the hematopoietic cells (26). However, due to its natural ability to synthesize and secrete large amounts of protein, the mammary gland has become the primary focus for the expression of heterologous proteins in large mammals. Transgene expression has been successfully directed to the mammary gland using promoter sequences from milk protein genes such as those encoding ovine β-lactoglobulin (BLG), goat β-casein, and murine whey acidic protein. The BLG promoter was used to direct expression of human α_1-antitrypsin in lines of transgenic mice and sheep (27). Interestingly, a wide variation in expression was observed between mouse lines, and from one lactation to another within a single line. In sheep however, similar high levels of heterologous protein were expressed in milk over consecutive lactations and over several generations in a given transgenic line, allowing the viable development of a flock of transgenic sheep. In separate studies high levels of expression of human tissue plasminogen activator were obtained in goat's milk under the control of the goat β-casein promoter (28). The development of suitable purification methods and the use of transgenically produced proteins in clinical trials are well advanced, and, if successful, will have important implications for the production of human proteins in transgenic livestock. Poor expression of the ovine promoter in the mouse may reflect species differences in recognizing heterologous versus homologous promoters and raises questions concerning the predictive value of mouse models. At best therefore the generation of transgenic mice may, in certain cases, only be a guide to the potential success of a transgene construct in another species.

Gene transfer could equally be used to enhance the quality and suitability of milk derived from domesticated animals as a food for human consumption. Human milk is devoid of β-lactoglobulin, which is responsible for most of the allergies to cows' milk, and has a relatively high content of lactoferrin, which is important in iron transport and combating bacterial infections. One could envisage in the future the reduction of saturated fat content in cows' milk and the knock-out of unwanted proteins or their replacement with other more useful components. Through the manipulation of milk constituents it should be possible to more closely emulate the desirable components of human milk. The alteration of milk composition would appear to be a practical possibility given that milk micelles are remarkably tolerant to changes in composition, as demonstrated by the knock-out of the mouse β-casein gene (29). Ethical concerns regarding the generation of transgenic animals, which have been engineered specifically for pharmaceutical, medical, or nutritional reasons, lie outside the scope

of this overview, however it must be clearly ascertained that expression of a transgene does not compromise the animal.

Xenograft organs for transplantation surgery

The shortage of human organs for transplantation has raised interest in the possibility of xenotransplantation, i.e. the use of animal organs (30). However, the major barrier to successful xenogeneic organ transplantation is the phenomenon of complement-mediated hyperacute rejection (HAR), brought about by high levels of circulating natural antibodies that recognize carbohydrate determinants on the surface of xenogeneic cells. After transplantation of the donor organ, a massive inflammatory response ensues through activation of the classical complement cascade. This leads to activation and destruction of the vascular endothelial cells and, ultimately, the donor organ. The membrane-associated complement inhibitors, endogenous to the donor organ, are species restricted and thus confer only limited resistance. The complement cascade is regulated at specific points by proteins such as decay accelerating factor (DAF), membrane cofactor protein, and CD59. These regulators of complement activation are species specific. The initial strategy used to address HAR in porcine-to-primate xenotransplantation was to produce transgenic pigs expressing high levels of the human terminal complement inhibitor, hCD59. This was shown to protect the xenogeneic cells from human complement-mediated lysis in vitro (31). More recently, organ transplantation has been achieved using donor pigs which expressed human DAF on their endothelium (32), or both DAF and CD59 on erythrocytes, such that the proteins translocated to the cell membranes of endothelial cells (33). After transplantation, the pig hearts survived in recipient baboons for prolonged periods without rejection (33). Clearly, such genetic manipulations are bringing xenotransplantation ever closer to reality. If the isolation of suitable ES cells and application of homologous recombination becomes a reality in the pig, it may be possible to knockout the antigenic determinants to which antispecies antibodies bind, as a further strategy for eliminating HAR.

Summary

The use of nonmurine species for transgenesis will continue to reflect the suitability of a particular species for the specific questions being addressed, bearing in mind that a given construct may react very differently from one species to another. The application of transgenesis in the pig should produce major advances in the fields of transfusion and transplantation technology, while alterations in the composition of milk in a range of domesticated animals will have major effects on the production of pharmacologically important proteins and could eventually lead to the development of human milk substitutes. Despite the lack of germline transmission to date, major efforts continue to be directed towards the generation and use of ES cells from nonmurine species, using both traditional and new technologies, and the availability of such cells is likely to accelerate both the use of such species and the precision with which genetic changes can be introduced.

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ELSEVIER

TRANSGENIC LIVESTOCK: PROGRESS AND PROSPECTS FOR THE FUTURE

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ABSTRACT

The notion of directly introducing new genes or otherwise directly manipulating the genotype of an animal is conceptually straightforward and appealing because of the speed and precision with which phenotypic changes could be made. Thus, it is of little wonder that the imagination of many an animal scientist has been captivated by the success others have achieved by introducing foreign genes into mice. The private sector has embraced transgenic livestock technology resulting in the formation of two new industries. However, before transgenic farm animals become a common component of the livestock production industry, a number of formidable hurdles must be overcome. In this brief communication, the technical challenges are enumerated and possible solutions are discussed.

Key words: transgenic livestock, gene transfer, microinjection

INTRODUCTION

The definition of transgenic animals is evolving. For the purpose of this paper a transgenic animal is one containing recombinant DNA molecules in its genome that were introduced by intentional human intervention. In this review I will focus on animals in which transgenes were introduced into preimplantation embryos by pronuclear microinjection, with the intended consequence of producing germline transgenics as opposed to somatic cell transgenics. Though there are other means of introducing genes into preimplantation embryos (20,29), pronuclear microinjection, basically as originally described by Jon Gordon (25), and as modified for livestock in our laboratory (65), is still the predominant method employed.

Acknowledgments

Many of the concepts, conclusions and visions of the future included in this manuscript have evolved over the years from discussions at our Friday afternoon lab meeting. Vern Pursel and Caird Rexroad, Jr., who pioneered transgenic livestock technology, provided the leadership. In recent years Ken Bondioli, David Kerr, Paul Hyman and Uli Tillmann have provided valuable new insights and new approaches that have and will advance the field.

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WHY MAKE TRANSGENIC ANIMALS?

A Medline search reveals that over 6,000 scientific articles have been published in which transgenic animals (mostly mice) were used to answer basic research questions. By contrast 289 papers dealt with transgenic livestock, of which 24% were reviews. The limited publication record for transgenic livestock species reflects the high costs and technical difficulties associated with producing transgenic livestock more than lack of applicability of this technology to farm animals. A number of well defined goals have been enumerated in the numerous review articles written by animal scientists. Not surprisingly, many of the proposed applications closely parallel the long term objectives of animal agriculture.

In theory, transgenic technology provides a mechanism by which economically important traits can be attained more rapidly than by selective breeding without concern of propagating associated, possibly undesirable, genetic characteristics. If genetic precision and speed of improvement were the only advantages of transgenic technology, use of that methodology might be difficult to justify. That is because current cost of producing transgenic animals are high and understanding of the appropriate genetic manipulations required to influence economically important traits is limited. However, transgenic technology offers much more. Genes can be transferred across species boundaries and can be modified to function very differently than they do in their native form (gene products, tissue specificity, and timing of expression can be altered). The ability to redirect expression of genes to another organ has spawned the transgenic bioreactor industry. For the most part, transgenic bioreactors are farm animals designed to produce new proteins in their milk or other body fluids. It is envisioned that this approach will have application in both food production and the biomedical arena. Modifying the composition of milk through genetic engineering is the topic of Dr. Bremel's paper in these proceedings and will not be dealt with here.

TRANSGENIC LIVESTOCK PROJECTS

For the sake of brevity, only a very brief summary of the 37 gene constructs that have been tested in livestock will be reported here. The reader is referred to two excellent reviews that list those constructs and their consequences (16,53).

The Transgene.

The power of transgenic technology is derived from the introduction of genetic information with new functionality. The strategy for building a transgene (fusion gene) involves selecting a genetic regulatory element (often called promoters, but usually containing both an enhancer element and a promoter) that will determine the tissue in which the gene is to be expressed and the time and magnitude of expression. In some cases, the regulatory element can act as a switch, allowing the transgene to be turned on and off at will. The second part of the gene construct consists of DNA sequence encoding the desired protein (often referred to

as the structural component of the transgene). For example, in the first transgenic livestock experiment (28) we wanted to increase the levels of circulating growth hormone in a controlled manner. The gene construct used to accomplish this consisted of the regulatory element of a metallothionein (MT) gene fused to the coding sequence for growth hormone (GH). Metallothionein is an inducible liver enzyme, and its gene is usually quiescent (turned off) until a threshold level of circulating zinc or cadmium triggers transcription. Therefore, it was expected that the MT-GH fusion gene would be silent until the animals were fed zinc. In those experiments GH expression could be inducted but, in most cases, the transgene could not be turned off completely. New more complex inducible approaches are now being tested (23,26). These new systems rely on tetracycline or its analogs to activate or repress transgene expression. It is too early to know if these strategies, in their current form, will be more tightly regulated then the MT system. However, if they are not, the general paradigm on which the new systems are based will probably lead to improved inducible systems

Agricultural Transgenic Projects.

The vast majority of original research reports have focused on growth enhancement. Growth hormone (GH) was the structural gene employed in 13 of those publications and the gene for growth hormone releasing factor in four. Other structural genes tested include IGF-1, cSKI and an estrogen receptor. The regulatory elements derived from MT genes, from various species, were most frequently used appearing in nine of the growth-related fusion genes. Long terminal repeats (LTR) from two retroviruses, MLV and RSV, and sequence from CMV, a DNA virus, served as regulatory components of transgenes, as have the promoters from albumin, prolactin, skeletal actin, transferrin and phosphoenolpyruvate carboxykinase (PEPCK) genes. All but two of 21 growth constructs were tested in pigs and the most striking phenotypes resulted from the use of MT-GH fusion genes (53).

Seven transgenes designed to enhance disease resistance and to produce immunologically-related molecules have been introduced into pigs and sheep (5,13,41,67). Though desirable expression patterns have been reported in several of the projects, none of the studies has progressed to the point of demonstrating a beneficial effect of transgene products.

Very recently it has been reported that transgenic sheep with enhanced wool production characteristics have been produced (9). The results are quite promising; if no unforeseen anomalies occur, transgenically produced wool maybe the first marketed livestock product.

Biomedical Transgenic Projects.

Other proposed transgenic farm animal applications are decidedly non-agricultural in nature. One of the first transgenic animal companies demonstrated the feasibility of producing new animal products by manufacturing human

hemoglobin in pigs, to serve as a principal component of a human blood substitute (59). Human antibodies have also been produced in transgenic mice (62). Another area where transgenic animals, especially pigs, will have a significant impact on society will be in the development of human genetic disease models. To date, genetic disease models have been generated in mice for atherosclerosis (6), sickle cell anemia (18), Alzheimer's disease (21), autoimmune diseases (44), lymphopoiesis (33), dermititis (55), and prostate cancer (61). These models for the most part require "knocking out" the function of a gene or replacing an existing gene with a mutant form. Many of these models will have to be replicated in farm animals to be useful. Unfortunately, the stem cell technology required to generate most of the disease models is still in development for livestock (51).

Finally, a new use not reported in the above mentioned reviews deserves note. The objective of this new endeavor is to genetically engineer animals, primarily pigs, so that their organs can be used as xenografts for humans. Preliminary studies to test the concept have been performed in mice (40,42) and transgenic pigs have now been produced (19,54). Though several strategies are being explored, the general approach has been to block activation of complement, which is normally part of the acute transplantation rejection response. These organs are intended for temporary use, until an appropriate human organ becomes available. However, as the technology develops, a driving force will be the design of transgenic organs for extended use or permanent transplantation.

CHARACTERISTICS OF TRANSGENIC ANIMALS

Transgenic livestock projects are costly, primarily because the process is inefficient. Production costs range from $25,000 for a single founder pig to over $500,000 for a single functional founder calf (64). The calculation for cattle was based on obtaining zygotes by superovulation of embryo donors, the normal practice for all mammalian species. However, the costs are reduced by as much as a third if oocytes derived from ovaries collected at slaughter are the starting material. The remainder of this review will be devoted to characterizing the transgenic animal model, to identify points in the process that reduce efficiency, and finally discussing possible approaches that have been proposed to overcome major hurdles to progress.

Transgene Integration.

Even though several hundred copies of a transgene are microinjected, any transgene that becomes incorporated into the genome generally does so at a single location. Exceptions are rare (58). Thus, transgenic founder animals are hemizygous for transgenes. It is also common for a transgene locus to contain multiple copies of the transgene arranged in a head-to-tail array. These two characteristics of transgene loci should provide clues to the mechanism by which transgenes integrate. So far, few researchers have formulated compelling hypotheses to explain the event (2,47) and the hypotheses that have been proposed remain untested

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Table 1. Examples of
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Species	Injected & transferred embryos (No.)	S
Mice	12,314	
Rabbits	1,907	
Rat	1,403	
Cattle[c]	1,018	
Pigs	19,397	
Sheep	5,424	

[a] Number of experiments.
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Without knowledge of the molecular mechanism it is going to be extremely difficult to devise approaches to make transgene integration more efficient.

Transgene integration efficiency is low and ranges from about 1% in farm animals (cattle, sheep and pigs) to about 3% in laboratory animals (mice, rabbits and rats, Table 1).

Table 1. Examples of embryo survival and transgene integration efficiencies from several laboratories.

Species	Injected & transferred embryos (No.)	Studies[a] (No.)	Offspring[b] (No.)	Transgenic animals produced: Per Offspring (%)	Transgenic animals produced: Per embryo injected & transferred (%)	Refs.
Mice	12,314	18	1847	17.3	2.6	(63)
Rabbits	1,907	1	218	12.8	1.5	(28)
Rat	1,403	5	353	17.6	4.4	(45)
Cattle[c]	1,018	7	193	3.6	0.7	(30)
Pigs	19,397	20	1920	9.2	0.9	(53)
Sheep	5,424	10	556	8.3	0.9	(53)

[a] Number of experiments, which in most cases was equivalent to number of different gene constructs tested.

[b] The value for cattle includes both fetuses and live born calves.

[c] Eleven thousand two hundred and six eggs were microinjected and cultured. One thousand and eighteen developed to morula or blastocysts and were transferred into recipient cows.

Transgene Expression

Even after the one in 33 to one in 150 injected and transferred eggs results in a transgenic animal the efficiency of the process is further diminished by failure of the transgene to be transcribed. Transgenes are expressed (transcribed) in only about half of transgenic lines, though some specific transgenes are expressed in a higher proportions (15-97). If a founder expresses its transgene, so do its transgenic offspring. It is not clear why some transgenes are expressed in all lines and others in only half the lines. Transgenes are sometimes activated in unintended tissues (ectopic expression), and timing of expression can be shifted relative to development. Our lack of understanding of essential genetic control elements makes it difficult to design transgenes with predictable behavior. The abbarent expression patterns (no expression or wrong expression) seen in some linese of transgenic animals has been attributed to the so-called "position effect." If a transgene lands near highly active genes, the transgene's behavior maybe influenced by endogenous genes. Other transgenes may locate in transcriptionally inactive (heterochromatin) regions. The transgene may function normally or be completely silenced by the

heterochromatin. It is likely that both of these factors (position effect and unidentified control elements) contribute to lack of transgene expression in some lines and variable expression in other lines. Some of these problems will be obviated by use of "boundary" DNA sequences that block the influence of surrounding genes (34,43). Refining transgenic technology for farm animals will remain a challenging task in part because experimentation will often have to be conducted in the species of interest. That is because transgene expression and the physiological consequences of transgene products in livestock are not always accurately predicted in transgenic mouse studies (28,48).

Transgene transmission.

Because founder animals are usually single integrant hemizygous for the transgene, one would expect 50% of their offspring to inherit a copy of the transgene locus. This is true for about 70% of transgenic founder mice (49). The remaining founders either do not transmit transgenes to their offspring or transmit transgenes at a low frequency (52,53). It is commonly thought that the non-Mendelian inheritance is the result of transgene mocasicism in germ cells. This could be caused by late integration of transgenes during embryonic development (60). It has been proposed that non-Mendelian inheritance patterns can also be caused by diminished fertilizing ability of transgene bearing sperm (17). The latter explanation may be a special case, because the thymidine kinase gene used in that study was inadvertently expressed in testes.

POTENTIAL SOLUTIONS FOR IMPROVING EFFICIENCY

Testing Transgenes.

Because the "rules" for transgene design are still vague, it is important to have a reliable system for testing gene constructs. The most cost effective method of characterizing the performance of a transgene is cell culture transfection studies. Unfortunately, such studies have a low predictive value (50). The next most cost effective method for testing gene constructs is production of transgenic mice, which as mentioned above do not faithfully predict a transgene's performance in livestock species. Nevertheless, a reasonable amount of useful information about transgene function can be derived from transgenic mouse studies. Currently, the only approach that yields truly informative data is testing transgenes in the livestock species of interest. This is obviously an unsatisfactory, time consuming, expensive testing option. One alternative approach that we are exploring is based on the fact that transgenes will function after being "shot" into somatic tissue. We have been focusing our efforts on the mammary gland, but almost any target organ should be amenable to this approach. We have recently demonstrated that both RNA and protein can be detected following introduction of transgenes into sheep mammary tissue, *in situ* (22,37). Once we confirm that "gene-gunned" transgenes function as they do in transgenic animals, this approach should dramatically reduce the costs and time of evaluating gene constructs.

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Improving Integration Frequency

From Table 1 it is clear that integration rates are lower for livestock species than for laboratory animals. Eggs of livestock species are more difficult to microinject than eggs of laboratory animals. However, competent microinjectors can reliably inflate pronuclei with DNA-containing solutions. Furthermore, integration problem occurs after the transgene is deposited. But timing of microinjection may contribute to differences in integration efficiency. It is thought that transgene integration occurs during DNA replication (2), so it would be advantageous to microinject before or during early S-phase preceding the first mitotic division. For the most part that is when laboratory animal eggs are microinjected, but microinjections are apparently performed during late S-phase or later in livestock species (for a full discussion see (63)). Efforts to inject *in vitro* fertilized bovine zygotes early have failed because of difficulties in visualizing pronuclei (K. Bondioli, personal communication and unpublished data). Efforts to synchronize microinjection and S-phase in bovine zygotes have thus far not been fruitful (24).

One way to insure that the transgene is in place before the first mitotic S-phase is to introduce the transgene at fertilization. That could be achieved by sperm-mediated gene transfer (4,38). Notwithstanding the controversy this approach has generated (8), it clearly represents an intriguing method that shows some promise (57). Accumulating evidence suggests that sperm of several species can bind transgenes (11,32,39,68) and carry the genes into oocytes where in some cases the gene persists (4,12,31). However, it appears that in almost all cases, the transgene DNA becomes rearranged or otherwise mutated by the process (Corrado Spadafora, personal communication). Another potential sperm-based delivery approach has been foretold by a pioneering study conducted by Ralph Brinster (7). In that study, transplanted spermatogonial cells generated sperm capable of fertilizing oocytes and offspring were produced. If a means is found to culture, transfect and select spermatagonia with transgenes, Brinster's transplantation scheme could be used to produce transgenic animals. Others have proposed directly transfecting testes as a means of transforming sperm (56).

Retroviral-mediated gene transfer is also a potentially alternative approach for introducing transgenes into embryos with high efficiency (29,36). Though the technique solves the low integration frequency problem, it creates other inefficiencies by generating mosaic founders that may not transmit their transgene. Furthermore, retroviruses can carry only a limited amount of exogenous DNA and therefore the technique limits the size of transgenes. If cDNA based transgenes, which are relatively short, were efficiently expressed, the transgene size restriction would not be a significant problem. However, many cDNA based gene constructs are poorly expressed in transgenic animals (66).

Selection of transgenic embryos.

With no obvious or immediate solution for improving integration frequency, what else can be done to increase efficiency of producing transgenic livestock? One of the most widely discussed approaches is selection of transgenic embryos before they are transferred to recipients (1,14,35,46). If transgenic preimplantation embryos can be identified by analyzing embryo biopsies with the polymerase chain reaction (PCR), the number of recipients required could be greatly reduced. For example in Dr. Bondioli's study ((30), Table 1), 1,018 bovine embryos were transferred into over 1000 cows resulting in seven transgenic calves and fetuses. If embryo selection had been possible, fewer than 20 recipients would have been required. Unfortunately, mounting evidence suggests that this approach will not work. In two very similar studies (10,14) microinjected mouse embryos were cultured to the 8-cell stage, and blastomeres were isolated and analyzed for the transgene by PCR. In our study (10) none of the 8-cell embryos had transgenes in more than 4 blastomeres. We speculate that immediately upon microinjection, transgene copies join to form multi-copy circular arrays. One of these arrays may eventually become integrated, while the non-integrated arrays segregate as daughter blastomeres are formed. If integration occurs after the one-cell stage, some blastomeres may not contain an array, even though the embryo is transgenic. The converse is also possible (all blastomeres acquire arrays but none integrate). Analysis of embryo biopsies could therefore be misleading.

Another scheme for selecting transgenic embryos before transfer is based on expression of a selectable marker-containing transgene. The preliminary results from two recent studies (3,60) appear to be promising. In both studies, transgenes containing a neomycin resistance gene (neo) were microinjected into pronuclei of mice (60) or bovine (3) embryos. The embryos were then cultured in the presence of G418, a neomycin analog, in the hope of killing embryos that did not express the neo gene. Because this approach is based on gene expression and because transgenes can be expressed without being integrated, embryos containing unintegrated copies of the transgene could survive the selection process. However, since G418 interferes with protein synthesis, the blastomeres that expressed the neo gene would have a developmental advantage over those that did not. Therefore, the blastomeres expressing the neo gene might divide more rapidly and have a higher probability of participating in the formation of the inner cell mass (66). Further studies will have to be conducted to determine if this scheme has merit.

In The Future

The tools for gene transfer are in hand, albeit the process is inefficient. Over the next decade, bioreactor and xenograft industries will mature and useful new products will be marketed. The value of possible products will drive the technology as funding for basic research from conventional sources becomes increasingly limited. Researchers will need to develop a better understanding of how mammalian genes are controlled, and identify key genes in regulatory pathways of

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phenotypic characteristics that are to be altered to bring the fruits of this technology to animal agriculture. There is a serious need to transfer transgenic animal technology from a few practitioners to many more laboratories worldwide. Progress in the field will be limited as long as the capabilities to explore this potentially powerful tool is only in the hands of a few. To entice other scientists, the efficiency of producing transgenic farm animals will have to be improved. But the horizon looks bright. Many recently trained animal scientists are now equipped with the knowledge and technical skills needed to advance this technology.

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THE USE OF TRANSGENIC ANIMAL TECHNIQUES FOR LIVESTOCK IMPROVEMENT

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INTRODUCTION

Breeding of livestock has always been characterized by selection of spontaneously occurring mutations which could be observed in the phenotype of the animals. This could be achieved even though the genetic and molecular mechanisms underlying those phenotypes were not understood. Depending on the generation intervals of the species involved, years to centuries were required to establish certain breeds in livestock fulfilling the needs of man. Still the ideal farm animals have not been found. As higher meat, egg or milk yields are obtained, higher susceptibilities towards environmental influences such as climate or food changes or stress are observed and disease resistance declines, especially when animals are kept in large numbers under industrial production conditions. Since the first successful gene transfer experiments into the germ line of mammals, namely mice, were reported in 1980 and 1981, many attempts have been made to use gene transfer techniques in livestock in order to improve the overall quality and productivity of farm animals.

Transgenic mice exhibiting the desired expression of the newly introduced gene and therefore showing an altered phenotype can now be produced routinely with different gene transfer techniques. Such mice are used in molecular biology research serving as genetically transformed, in vivo, models elucidating different kinds of scientific problems. In contrast, only little progress has been made in recent years in producing transgenic mammals of other species. The major drawbacks are inadequate regulation of expression of the foreign genes and therefore the desired phenotypical improvement has not been obtained so far.

In this chapter we will describe briefly the basic techniques involved and summarize the results which have been obtained until now, in respect to the production of transgenic mice and livestock in particular. The difficulties encountered in the application of

gene transfer techniques, which have been successfully used in mice, to farm animals, are discussed and their future implications for animal breeding are outlined.

PRODUCTION OF TRANSGENIC MICE

The term "transgenic" was introduced originally by Gordon and Ruddle (1) for mice which had integrated a foreign gene into all somatic tissues examined. This had been achieved by injecting these genes into the pronuclei of fertilized mouse ova. Since it became evident later (1a) that the offspring of these mice can inherit the foreign genes in a Mendelian fashion, the word transgenic is now commonly used to refer to a stable germline integration of foreign genes. At present three different approaches have been reported which succeed in the establishment of transgenic mouse lines. These methods include pronuclear injection of DNA, infection of embryonal stages with recombinant viral vectors and the production of germline chimeras which consist partially of totipotent, genetically transformed, cell lines.

Pronuclear Injection

Several groups (1a-4) established this method of gene transfer simultaneously showing that by using this technique integration of the introduced foreign gene into all somatic and also into the germ cells of the developing animal can be achieved. In order to achieve optimal results, approximately 1 pl of a DNA suspension containing 200 to 2000 linearized copies of the foreign gene is injected into either pronucleus of fertilized mouse eggs (5). After overnight cultivation cleaved eggs (50% to 75%) are transferred to the oviducts of foster mothers. Of these 10% to 25% usually develop to term and about 15% to 37% of the young born inherit the injected genes, which leads to an overall efficiency rate of 1% to 4% based on the total number of zygotes manipulated.

Provided the introduced genes are integrated into the genomic DNA before the zygote enters cleavage, all embryonal cells will contain the same integration site and copy number of the foreign gene. Integration after the first cleavage will give rise to chimeric animals, which consist of at least two genetically different cell populations. According to Wilkie et al. (6) the latter seems to be true in at least 30% of the mice born after microinjection of DNA.

Infection of Embryos by Viral Vectors

Infection of blastocysts by SV40 (7) was the first evidence that foreign genes could be integrated into the genome of embryo-

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nal cells and lead to their stable integration into somatic tissue. Jaenisch (8) also observed that the M-MuLV provirus was not only retained by somatic cells after embryonal infection but was also transmitted to the offspring of the resulting mature mice.

Viral infection of the preimplantation embryo is naturally prevented by the existence of the zona pellucida. This barrier can be overcome by either enzymatic digestion of the zona, so that two-cell to morula stages can be infected (9,10) or direct microinjection of virus particles into the blastocoele cavity (7). This procedure has been used successfully to introduce proviruses into the germline of mice. However, the resulting embryos generally are mosaic, as different integration events can take place in a variable number of embryonal cells (11).

To transfer foreign genes into the germline of mice, recombinant retroviral vectors can be used which contain the gene of interest. Since recombinant retroviruses generally are replication incompetent, they require the presence of helper virus for propagation. Stuhlmann et al. (12) have thus infected day-nine mouse fetuses with recombinant and helper virus simultaneously and observed subsequent integration and expression in the resulting

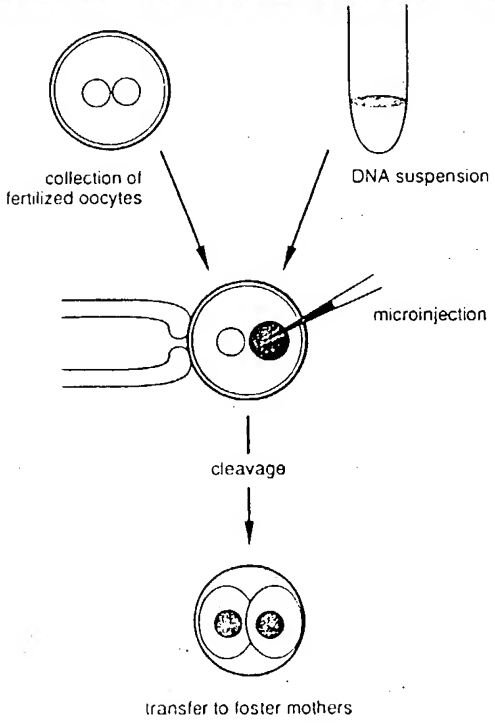

Figure 1. Pronuclear injection of DNA.

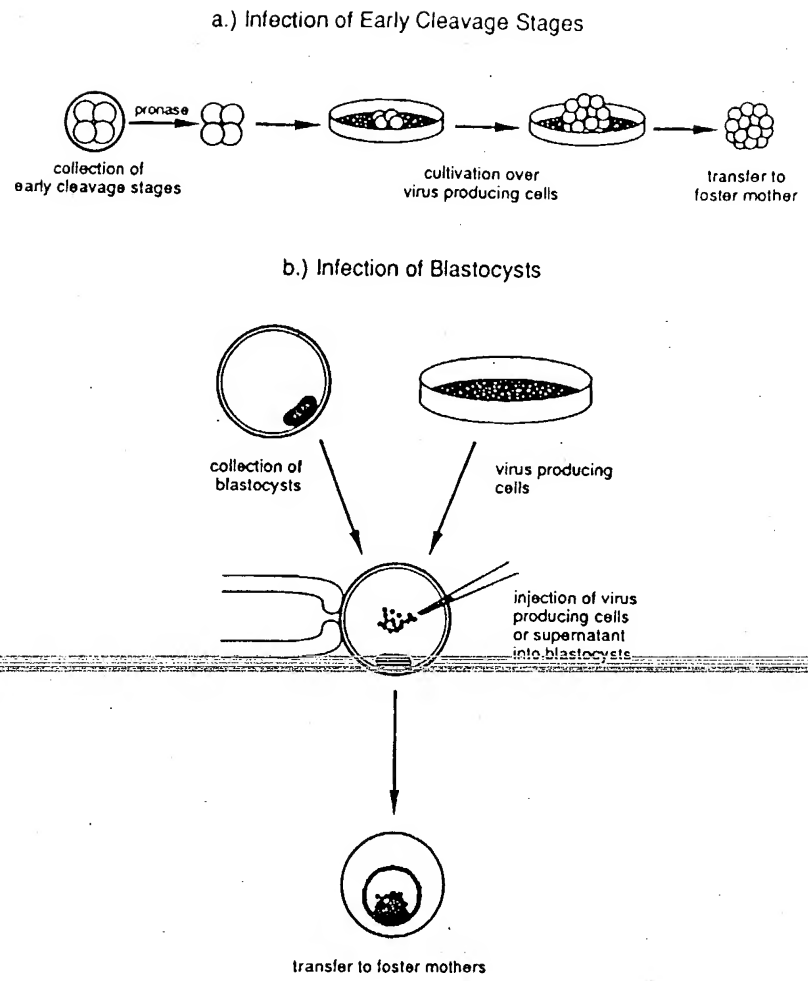

Figure 2. Infection of embryos with viral vectors.

offspring. However, their results suggested that the superinfection with helper virus in these mice had interfered with the spread of the recombinant virus and therefore limited the number of transformed somatic cells.

As separation of recombinant and helper virus yet cannot be achieved, an alternative propagation method using psi-2 cells (13) can be chosen if viremia from the helper virus is to be avoided in the resulting animals. Psi-2 cell lines have incorporated a retroviral genome which cannot be packaged into virus particles

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Van der Putten et al. (14) and Rubenstein et al. (10) have infected pre-implantation mouse embryos with recombinant replication-incompetent retrovirus without the use of helper virus. 197 denuded 8-cell stages cultivated over psi-2 monolayers for 16 hours and subsequently transferred to foster mothers gave rise to one animal which had incorporated the recombinant provirus including the foreign gene and transmitted it to its offspring (14). Rubenstein et al. (10) co-cultivated 278 4-cell-stage mouse embryos over psi-2 cells and obtained 76 (30%) live fetuses after transfer, of which one contained the recombinant provirus.

Production of Germline Chimeras

Chimeras can be produced either by aggregating two embryos or by injecting embryonal cells into expanded blastocysts, a procedure that results in the formation of individuals which consist of two or more genetically different cell lines (15). When totipotent embryonal cell lines, which can be grown in culture for many generations, are genetically transformed before being used for chimera formation, this technique offers another route of producing transgenic animals, provided that the transformed cells will participate in the formation of germ cells.

The first embryonal cell line used for this purpose consisted of murine embryonal teratocarcinoma (EC-) cells (16). However, these cells appeared to have the disadvantage of showing a tendency to lose their euploidy during in vitro cultivation and therefore lose their totipotency, especially to contribute to the germline. Also the development of abnormal fetuses was observed in EC-cell derived chimeras (17). More stable results can be obtained with embryonal stem ES-cells (18), which can be isolated from in vitro attached mouse blastocysts and grown in culture for many passages. After microinjection of ES-cells into expanded mouse blastocysts, Bradley et al. (19) obtained an average birth rate of 70%; about 50% of the young born proved to be chimeric, of which 20% also showed germline chimerism. Stewart et al. (20) aggregated 8-cell-stage mouse embryos with ES-cells and received birth rates of 36%, 20% of the pups being chimeric. The successful use of genetically transformed ES-cells (20,21) led to the expression of the transferred genes within somatic tissues of the chimeric animals. Robertson et al. (21) injected 10 to 12 ES-cells, which had been repeatedly exposed to psi-2 cells for transfection, and selected for transformation, into expanded mouse blastocysts. Out of 21 mice born after transfer 20 proved to be chimeric, of which two were reported to have transgenic offspring.

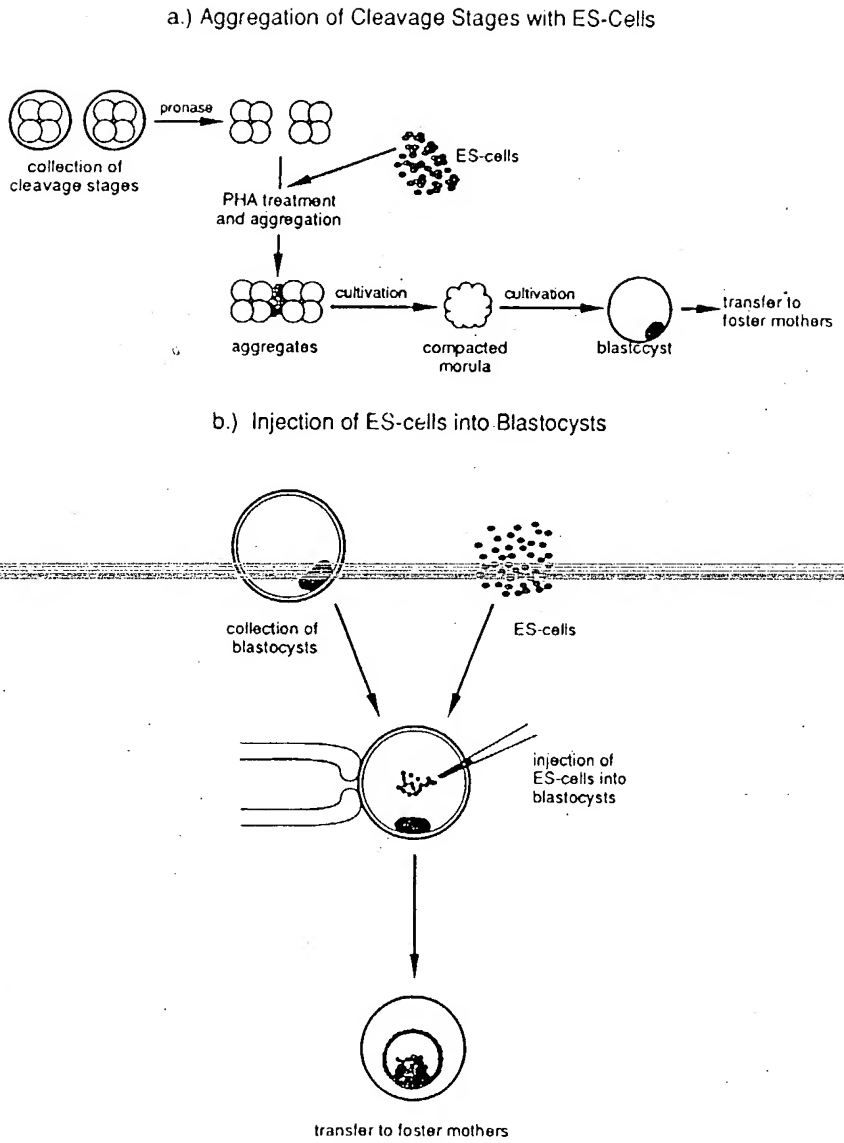

Figure 3. Production of chimeras with transformed totipotent cells.

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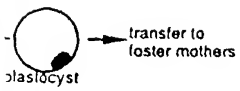

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According to Evans (22) 20% to 30% germline chimerism can be achieved by this method.

A very critical step in this approach of producing transgenic animals is the successful transformation of the ES-cells. There are several methods of introducing foreign genes into the genomes of these cells with variable degrees of efficiency and technical equipment and skill required. The calcium-phosphate-precipitation method, which is comparatively simple, yields only very few transformants (frequency 10^{-6} to 10^{-7}), but can be used if large numbers of totipotent cells are available (23,24,24a).

One alternative method of transforming EC- or ES-cells more efficiently is the use of recombinant retroviruses (20,21,25). Rubenstein et al. (25) infected EC-cells with psi-2 cell propagated recombinant retrovirus and demonstrated that 1 of 250 EC-cells had incorporated the retrovirus without any further multiplication of the virus in these cells. The efficiency of this retrovirus-mediated transformation method can be further increased depending on the infectivity and the titer of the recombinant virus as well as on the time of exposure (21).

If retroviral infection cannot be established for some reason, microinjection of DNA into the nuclei of totipotent embryonal cells offers a third most effective but cumbersome method of gene transfer with a frequency of 10^{-2} to 1 (24).

Genomic Integration of Foreign DNA in Transgenic Mice

The integration of foreign DNA introduced physically into the mammalian genome is up to now not clearly understood and appears to be an entirely random process. Experiments with mammalian cell lines have shown that after DNA transfections (calcium phosphate precipitates), transgenomes can be found within the nucleus, i.e., high molecular weight structures containing DNA, which are not associated with chromosomes (26). The stability of the genetic transformation process depends on its subsequent chromosomal integration during the following cell cycles (27). A number of observations (23,28-31) have shown clearly that foreign DNA, once within the nucleus, undergoes rather complex biochemical processing including ligation and recombination reactions. The importance of nuclear enzymes for the integration of foreign DNA into the genome has also been indicated by the experiments of Giulotto and Israel (32), who found that the transformation efficiency in synchronized mammalian cells was significantly higher when the experiments were performed during the early S-phase of the cell cycle.

In DNA-mediated gene transfer experiments with somatic mammalian cells, as well as after microinjection of foreign DNA into nuclei or pronuclei, the same integration pattern can be found. This pattern consists of only a single, rarely, two (33,34), integration sites per cell and the integration of one to 400 copies

(35) per site in a concatamer structure, i.e., a head-to-tail arrangement of the foreign gene copies.

Smithies et al. (36) have shown that homologous recombination can be used to target the integration site of foreign genes into endogenous gene loci of somatic mammalian cells. Until now homologous recombination of foreign DNA with endogenous genes has not been reported in transgenic mice. It may well be that this is due to the very low frequency of such events (10^{-9} in somatic cells), which makes it rather improbable to occur in only a few thousand microinjection experiments.

Usually intact gene copies are found to be integrated into the genomes of transgenic mice showing the expected restriction patterns in a Southern blot (1). It has been reported (1,3,37-44) that occasionally partially deleted or recombined foreign genes were integrated into some transgenic mice. Moreover, in established transgenic mouse lines instability of the transgenes is sometimes observed. This is expressed either in a reduction of the number of integrated exogenous gene copies (45) which Gordon (35) explains by their possible recombination and subsequent excision from the chromosomal structure, or in a change of their restriction patterns (47,48). The latter can be found within different generations (40,48) or even in ontogenetic development (47), indicating that structural alterations of the integrated foreign genes can occur during replication and meiosis.

Recent reports (44,49) suggest that alterations of the foreign genes introduced into the genomes are generated by extensive rearrangements of endogenous sequences flanking the integration sites. Tarantul et al. (44) hypothesized that repeated endogenous sequences, which are often associated with genome rearrangements, may be involved in the integration process of foreign DNA.

The biological gene delivery system by retroviral vectors differs from the physical procedures, since the virus itself induces mechanisms which lead to the integration of a biologically functioning provirus. It is important to emphasize that therefore this system also has its limitations, as these biological mechanisms regulating the expression of proviral genes may well interfere with the expression of the enclosed foreign gene and its regulation. This will be discussed later.

Foreign genes enclosed within a retroviral vector integrate as a single copy per integration site (8,9). Depending on the infectivity and the titer of the virus, multiple integration sites (up to 26) per cell can be obtained in ES-cells and, therefore, in transgenic mice (21). Although the instability of recombinant retroviral genomes in somatic and ES-cells (20) and also partial instability of M-MuLV in two provirus containing mouse strains (11) have been reported, no such evidence has been found so far within transgenic mice established by the use of replication deficient recombinant retroviral vectors. However, the comparatively small number of mice obtained so far by this method does not exclude the possibility of such events to occur.

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Expression of Foreign Genes and its Regulation in Transgenic Mice

Expression of foreign genes after pronuclear injection was first detected by E.F. Wagner et al. (3) in fetuses and (2) in living mice and their offspring by T.E. Wagner et al. (1a). Although several transgenic fetuses or living young were analyzed by these authors, only a few showed detectable expression of the foreign gene. Lack of expression was then explained by gene integration into inappropriate genomic sites, a theory that was supported by Palmiter, Chen and Brinster (54) and Lacy et al. (51), who observed that varying expression of foreign genes in transgenic mouse lines was accompanied by different methylation patterns (54) or abnormal chromosomal positions of these genes (51). Furthermore, it was pointed out that the amount of foreign mRNA produced in expressing transgenic mouse lines was not correlated with the copy number of the integrated gene (52), an observation which also indicated a possible role of the integration site in expression of transgenes. Recently, variance in expression was also discussed as a relative instability of mRNA molecules transcribed from the foreign gene construct (53).

Additionally, other factors have become known to be involved in the regulative mechanisms which determine the expression of foreign genes. The expression rate was drastically reduced when foreign genes had been integrated together with plasmid vector derived sequences (5,37,40), suggesting an inhibitory effect of prokaryotic sequences on foreign gene expression in transgenic mice.

The work of Palmiter and colleagues (52,54,55) has shown clearly that the expression of exogenous structural genes can be stimulated when these are fused to certain cis-acting factors, as exemplified by the mouse metallothionein (Mt-I) promoter. Rat or human growth hormone genes fused to this promoter were expressed ectopically in a variety of tissues under the regulation of the Mt-I promotor, which led to a striking increase in hormone production and growth rate of the transgenic mice. Moreover, Swanson et al. (56) have found in their experiments that expression of a Mt-I-rGH fusion gene in separate transgenic mouse lines was unaffected by the integration site, suggesting that certain gene constructs, which include appropriate cis-acting factors, can override other inhibitory environmental effects, thus decreasing the importance of the integration site to a certain extent.

Since then, transgenic research has mainly focused on the development of fusion genes which contain the information necessary for tissue specific and developmentally regulated expression patterns. Although eukaryotic gene regulation, especially in mammals, remains unclear in many respects, in particular when the involvement of cell specific trans-acting factors is considered, some cis-acting elements have been found to provide tissue specific expression in transgenic mice when fused to structural genes. Some examples in which gene expression was developmentally regulated and strictly directed into promoter specific tissues are listed in

Table 1

Promoter Specific Expression of Foreign Genes in Transgenic Mice

Promoter	Structural gene	Reference
elastase 1 (rat)	elastase I (rat)	57
elastase I (rat)	growth hormone (human)	58
myosin-L-chain (rat)	myosin-L-chain (rat)	39
beta-globin (human)	beta-globin (human)	59
beta-globin (mouse)	beta-globin (human)	60
insulin II (rat)	SV40-T antigen	61
alpha-A-crystallin (mouse)	CAT (prokaryotic)	62
alpha-I-collagen (mouse)	CAT (prokaryotic)	43
insulin (human)	insulin (human)	63 64
skeletal muscle actin (rat)	epsilon-globin (human)	40
whey acidic protein (mouse)	Ha-ras oncogene (human)	53
delta-crystallin (chicken)	delta-crystallin (chicken)	65
alpha-I-antitrypsin (human)	alpha-I-antitrypsin (human)	66
pancreatic amylase (mouse)	pancreatic amylase (mouse)	67

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Table 1.

In some cases the inducibility of expression of foreign genes in transgenic mice was reported, as for Mt-I fusion genes by heavy metals (52,54,55), a mouse MHC-II-antigen by interferon (68), the chicken transferrin gene by estrogens (69), the human insulin gene by glucose (64), the mouse pancreatic amylase gene by insulin (67) and whey acidic protein (WAP)-promoter fusion genes by lactogenic hormones (53). These results demonstrate that certain genes or gene constructs contain sufficient cis-acting information so that, after integration into the mouse genome, tissue specific and hormonal controlled expression can be obtained. However, other investigators (33,56,70,71) have shown that expression patterns of Mt-I promoter fusion genes in transgenic mice were not exclusively dependent on the promoter sequence itself, but that depending on the particular gene construct used, different expression patterns were found, suggesting internal cis-acting regions within the different fusion genes.

Expression of foreign genes can be regulated in order to compensate, to some extent, for certain endogenous insufficiencies in transgenic mice (41,72-74). These results indicate the possible application of gene transfer for germline gene therapy in animals.

When recombinant retroviral vectors are used, expression is complicated by mechanisms which regulate the expression of the provirus. Jaenisch et al. (9) have found the expression of retroviral genomes to be blocked when infection had occurred during pre-implantation mouse development, a phenomenon which also was observed in EC-cells (75). In some mouse substrains containing the M-MuLV provirus in their germline, activation of the viral genome can occur during embryogenesis, whereas in others it is not activated at all or at different times (11). The authors explained this finding by proviral integration into different genomic sites in each substrain. Later the expression block in embryonic cells was found to be correlated with de-novo methylation of the provirus after integration (75-77), but it has been doubted that this is the cause of the expression block but rather its consequence (78).

Linney et al. (79) and Gorman et al. (80) presented evidence that retroviral expression in EC-cells is impaired because of inappropriate functioning of cis-acting sequences within the M-MuLV LTR. The existence of trans-acting factors in embryonal cells which interact with these sequences has been postulated (80,81). When foreign genes introduced into recombinant retroviral vectors are transferred to mouse embryos or EC/ES-cells, the described expression block can be overcome when strong internal promoters are placed in the vector construct (10,20,25) or the LTR is partially deleted and replaced by other enhancers (14).

USE OF TRANSGENIC MICE IN BIOLOGICAL RESEARCH

Transgenic mice offer the unique possibility to study the developmental control of expression of introduced gene copies. The transferred and monitored genes can either be naturally occurring sequences or recombinant gene constructs. Since expression studies in vitro with eukaryotic cell lines are only of limited value in explaining the natural mechanisms involved in gene expression, many questions can only be answered after gene transfer into intact developing organisms. For example, transgenic mice have been successfully used to approach different problems in oncology. The c-myc oncogene was shown to be carcinogenic in mice when fused to a viral promoter (82,83) or immunoglobulin enhancers (84,85). Quaife et al. (86) induced pancreatic neoplasia in transgenic mice by fusion of the activated c-ras oncogene to regulatory elements of the rat elastase-I gene, whereas the c-myc gene was not capable of transforming pancreatic cells under these conditions, demonstrating the tumorigenic ability of the activated cellular oncogene. Palmiter et al. (86a) found that a single viral protein, namely the T antigen of SV40, was able to transform choriod plexus epithelia into malignant tumor cells. Sinn et al. (34) presented evidence for the synergistic tumorigenic action of the two oncogenes v-Ha-ras and c-myc when transgenic mice derived from different lines each containing one of these oncogenes fused to a MMTV-promoter were mated and their offspring analyzed.

Since it was shown that subunits of immunoglobulin genes can be introduced into transgenic mice (87) and their subsequent expression in B-cells was observed (88), several authors (42,89-95) have concentrated on transgenic mice, investigating the maturation of B-lymphocytes as a rearrangement process of immunoglobulin genes. Their observations lead to the confirmation and specification of the "allelic exclusion" theory (89).

Genes coding for a variety of different cell surface antigens have been introduced into transgenic mice and their functional expression reported (68,96-100), resulting in a better understanding of the function of these antigens. Other investigators (101-103) have used transgenic mice to investigate the mechanisms of hepatitis B virus surface antigen expression, uncovering interesting aspects of the pathogenesis of hepatitis B. Small and collaborators (104,105) established transgenic mice containing early regions of human papova viruses which resembled the pathological findings in humans and thus provide suitable animal models for the study of these diseases.

Work with transgenic mice containing different forms of beta globin genes (106,107) has revealed interesting information on the developmental regulation of beta globin synthesis. Recently, the use of transgenes as an autosomal DNA marker has served to elucidate the meaning and the mechanism of parental imprinting in transgenic mice (108,109).

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ATTEMPTS

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ATTEMPTS TO PRODUCE TRANSGENIC LIVESTOCK

Up to the present the production of transgenic rabbits (113-116), sheep (114,117), pigs (113,114) and chicken (118) has been reported. Attempts to produce transgenic goats (119) and cattle (120-122) have not so far been successful.

The main purpose of these experiments was the introduction of growth hormone genes fused to the Mt-I promoter into the germline of livestock in order to increase the growth rate of the animals (113-115,117,119,122). Viral TK genes or simian alpha-interferon genes have been injected into cattle embryos, the latter procedure aiming at the amplification of interferon production and therefore increased disease resistance in the animals (120,121). A fusion gene consisting of the rabbit uteroglobin promoter and the marker gene CAT was introduced into the rabbit genome in order to study the uteroglobin-specific expression mechanisms in more detail (116). In all the mammalian species pronuclear or nuclear (two-cell stage embryos) injection was performed. In chicken infection of recombinant and helper virus was achieved by injection of virus into the yolk of fertile eggs. To facilitate pronuclear or nuclear injections in pigs and cattle ova had to be centrifuged prior to microinjection in order to remove the ooplasmic granula which otherwise interfere with the microscopic visualization of the nucleus (114,120). Fluorescent staining of pronuclei as an alternative (123) has not been found to give satisfying results (114).

Birth rates after micromanipulation and transfer of rabbit ova to foster mothers are consistently 9.5% to 12% (113-116). Integration rates varied in this species between 5.5% (113), 12.8% (114), 24% (115) and 41% (116). The latter results were explained by gene injection during the DNA replication phase of the pronuclei; however, these were associated with a high mutation frequency of the transferred gene so that at least 60% of the transgenic rabbits had integrated structurally altered genes as shown by Southern-blot hybridization. Although several transgenic breeding lines of rabbits containing Mt-hGH fusion genes have been established, none

of these is consistently expressing the foreign gene or showing increased growth rates (124).

The production of one single transgenic lamb after manipulation of 1032 (114) and 436 (117) sheep ova, respectively, has been reported. One lamb had integrated a structurally altered gene (114) and neither one showed any expression of the foreign gene. Recently, the birth of 2 Mt-I-bGH transgenic and 9 Mt-I-hGHRF transgenic lambs has been reported (124). One of these expressed the bovine growth hormone gene and two expressed the gene for the human GH-releasing factor, but none of them showed increased growth.

Transgenic pigs have been produced with the use of growth hormone genes fused to the Mt-I promoter (113,114). Birth rates varied between 5.5% (113) and 9% (114). Brem et al. (113) reported 1 piglet out of 15 born to be transgenic, whereas Hammer et al. (114) obtained 20 transgenic piglets with an integration rate of 10.4%. Six transgenic pig lines have been established containing Mt-I-hGH fusion genes of which only one is expressing the foreign gene (124). These pigs show detectable amounts of hGH (human growth hormone) in their serum but do not grow significantly larger than controls. However, the average backfat of these pigs is less as compared to controls at a body weight of 90 kg indicating some effect of the exogenous GH on body composition (124).

Fabricant et al. (119) transferred 153 goat ova which had been injected with Mt-I-rGH (rat growth hormone) genes to 27 recipients. Of these 20 returned to heat and two aborted their pregnancies; the remaining recipients gave birth to 9 young of which 5 were stillborn. None of the remaining 4 kids which were analyzed proved to be transgenic.

Gene transfer experiments on cattle ova were first described by Lohse et al. (120) who injected about 1000 copies of the plasmid pMK containing the HSV-TK gene into pronuclei of centrifuged cattle oocytes. After in vitro culture for 24 hr about 30% of the manipulated embryos showed expression of the HSV-TK gene. Loskutoff et al. (121) injected HSV-TK or simian alpha-interferon genes into pronuclei or nuclei of bovine one-or two-cell stage embryos. After transfer of 81 manipulated embryos to 24 recipients 3 pregnancies were diagnosed. McEvoy et al. (122) transferred Mt-I-rGH genes or a modification of this gene carrying bovine papilloma virus enhancers to bovine one or two-cell stage embryos. Subsequent transfer of 47 manipulated ova to 17 recipients resulted in at least 4 pregnancies. The birth of transgenic calves has not yet been reported.

Transgenic chicken lines were obtained by retroviral infection of embryos (118). Out of 37 viremic males tested 9 were mosaic according to the integration of provirus into the germline. The frequencies of provirus transmission to their offspring varied between 1% and 11%. Animals from the F-1 generation transmitted the gene in a Mendelian fashion to the F-2 generation. These experiments aimed at the establishment of a suitable gene delivery sys-

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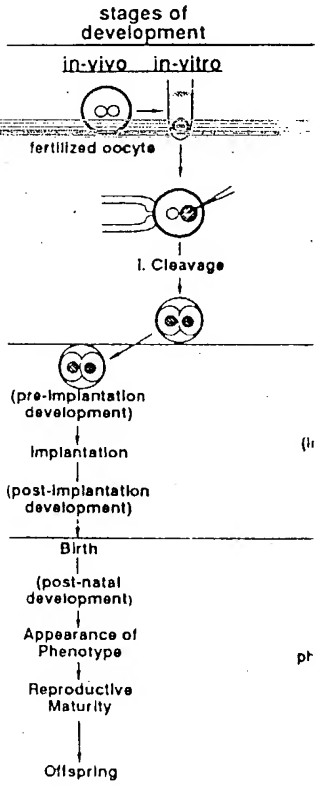

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Factors Limiting the Production of Transgenic Livestock

In summarizing the results mentioned above, it can be said that gene transfer into the germline of livestock is possible but the intended phenotypic improvement has not been obtained so far. However, results from transgenic mouse work suggest that this also will be possible as soon as more information on species-specific conditions can be acquired. Thus further experiments with livestock appear to be necessary. Unfortunately, production of trans-

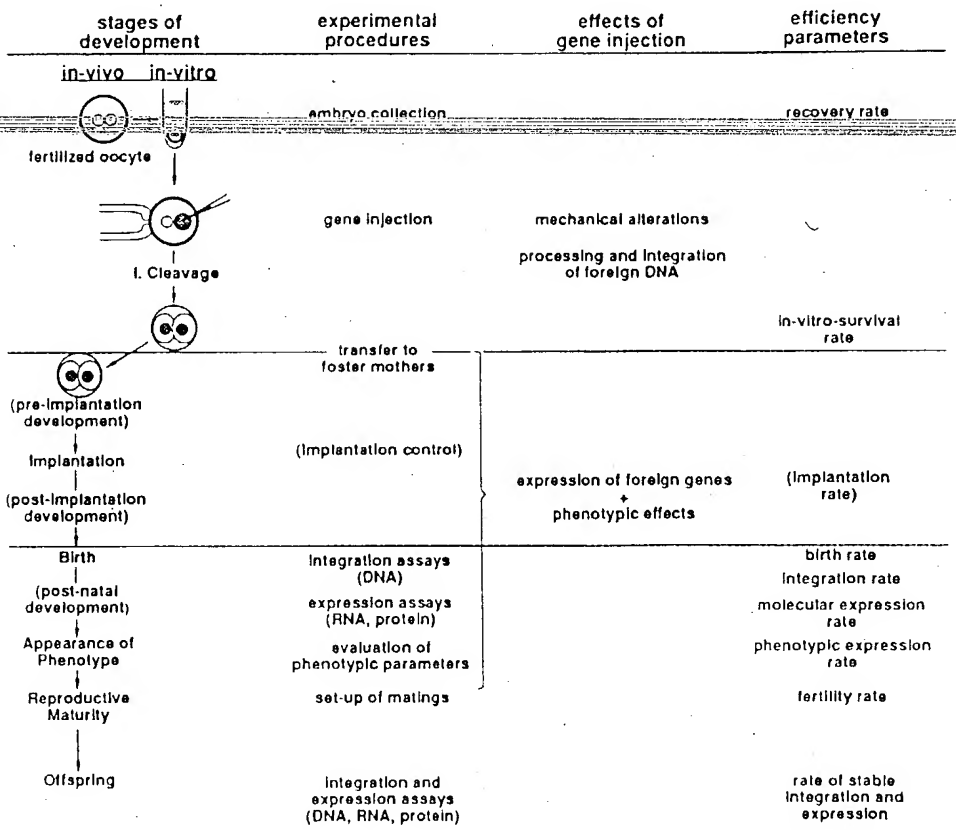

Figure 4. Steps in the production of transgenic animals (pronuclear injection method).

genic livestock is facing two major obstacles: first, very high costs for farm animal husbandry combined with long generation intervals restrict research in this field and second, a relatively small amount of scientific knowledge of farm animals is available as compared to the mouse as the major laboratory animal. How can these obstacles be overcome?

Successful gene transfer to the germline of farm animals requires the collaboration of three major branches of science. These branches are animal physiology, molecular biology and embryology. The latter provides the techniques for introducing foreign genes into the germline of the animals and therefore influences the efficiency rates of gene transfer. Molecular biology contributes the genes and monitors the molecular effects on the genome. An underestimated aspect in this field of research is the necessary participation of animal physiology which provides insight into the regulative mechanisms and phenotypic effects of functional proteins in different species. In order to analyze the current limitations of gene transfer to livestock the different steps of these experiments have to be considered (see Figure 4). Efficiency can be determined at different levels, each referring to the competence of the different scientific branches named above. First, the overall efficiency of the production of transgenic animals (transgenic animals/manipulated oocytes), second the expression efficiency and third, the efficiency of phenotypic improvement can be analyzed.

Considering the costs of livestock husbandry, it is obvious that the efficiencies should be as high as possible. From the data shown above it can be concluded that the maximal overall efficiency in producing transgenic animals is approximately 4% in mice (33,71), 3% in rabbits, (116) 1% in pigs (114) and 0.2% in sheep (117). In order to produce transgenic farm animals for research purposes this efficiency has to be increased drastically considering the expenses involved but until now hardly any attention has been paid to transgenic production efficiencies and little is known about the limiting factors. Overall efficiency is influenced by two components, the ratio of manipulated oocyte development to live young and the integration frequency of the foreign gene. Survival rates have been determined at different stages of in vitro or in utero development and after birth. In vitro cultivation results show that about 10% (1a) to 50% (2) of the manipulated mouse oocytes are lost at the one-cell stage. As these losses are directly due to mechanical alterations of oocyte structures, the varying results indicate the importance of technical skill required in these experiments. However, species-specific differences in mechanical susceptibility of oocytes exist; mouse oocytes, for example, are much more fragile than rabbit oocytes (unpublished observations). When pronuclear injections are performed in rabbit oocytes about 5% to 10% undergo lysis within a period of one hr and 83% cleave to two-cell stages (controls 100%) within 24 hr of in vitro cultivation (116). In

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vitro cultivation experiments over a period of 4 days subsequent to gene transfer to mouse ova resulted in a decrease of development to the morula stage of at least 20% (5). In these experiments different DNA concentrations were used. It was shown that the injection of buffer alone did not increase the survival as compared to the injection of 5, 53 or 520 gene copies. But when 5,300 gene copies were injected a further reduction of the development of 35% was noticed. Strojek (116) reported that two-cell stage rabbit embryos derived from ova injected with 2000 to 4000 gene copies showed a significant reduction in the in vitro development to blastocysts (75%) as compared to controls (89%). Rexroad and Wall (125) investigated several factors which can interfere with the developmental potential of injected sheep ova. They found that after transfer to sheep oviducts, in vivo development of non-injected ova to 32-cell stages was impaired by in vitro cultivation for four hours alone (65% as opposed to 89% in immediately transferred ova). Additional microscopic inspection for 30 min did not lead to further impairment of the survival, but injection of Tris/HCl buffer was detrimental (42% survival). After injection of gene suspension only 19% of the ova developed to 32-cell stages in vivo. These consistent results indicate that the gene suspension buffer as well as the foreign genes themselves can exhibit toxic effects on the developing embryos. Furthermore, birth rates (young born/eggs transferred) of 10% to 30% are usually found in a large number of experiments when transgenic mice are produced. In contrast, birth rates of 50% to 60% can be obtained after transfer of unmanipulated mouse oocytes (unpublished observations). Thus it can be concluded that not only technical insufficiencies are responsible for these low results after gene injection but that they rather reflect a further reduction in the developmental potential of the manipulated oocytes. This assumption is supported by the observation that after gene injection into rabbit oocytes, implantation was low (30% of non-injected) and post-implantational resorption rates exceptionally high (50%) in two independent sets of experiments (116). Additionally, viability of the animals born appears to be reduced in mice (90,97), rabbits (115,116,124) and pigs (124,126). These independent observations consistently suggest deleterious effects of pronuclear gene injection on the viability of embryos which lead to impaired pre- and post-implantational as well as post-natal development rates. These effects probably cannot be explained by mechanical alterations alone.

Low integration frequencies also limit the efficiency of gene transfer to livestock by pronuclear injection. In mice the integration frequency was increased to 37% if higher DNA concentrations were used (5). But this was associated with lower viability of the manipulated embryos, so that the overall efficiency was limited to approximately 6% (d-13 fetuses analyzed). It is still unknown whether integration frequency of foreign genes initially is higher in pre-implantation embryos and is reduced subsequent to

integration by contra-selection; however, at maximum 24% of the rabbits born (115) and 10.4% of the born piglets (114) have integrated foreign gene copies. Strojek (116) has attempted to increase this rate by restricting the time of gene injection to the pronuclear DNA replication phase, referring to results of Giulotto and Israel (32) who had worked with synchronized somatic cells. As a result gene integration was found to be highly increased (integration rate 41%), but this was accompanied by a high frequency of mutation of the integrated foreign gene. These findings were considered to be related to species-specific activities of pronuclear enzymes, as rabbits show much faster pronuclear development than mice, for example, in which similar observations have been reported only rarely (see section Genomic Integration of Foreign DNA in Transgenic Mice).

It can be assumed that genetic and species-specific conditions can cause different limitations of gene integration efficiency, an assumption that is supported by the fact that in sheep integration frequency is comparatively low (114,117) and also by the observation that integration frequency varies considerably between different mouse strains (5) and somatic cell lines of different species (127). Therefore, the major limitations of gene transfer efficiency into the germline of animals appear to arise from deleterious effects of the injected gene suspension on the viability of the manipulated embryos and from low integration frequencies. As both phenomena are as yet poorly understood, considerable improvement in the efficiency of this gene transfer method appears to be rather unlikely in the near future. It would, therefore, be of great advantage if genetically pre-selected totipotent cells could be used to produce germline chimeras which contain the desired genetic make-up in their germ cells, including intact and stably integrated foreign gene copies. This procedure has been shown to be highly efficient in mice (21) and experiments are under way in our laboratory to establish this technique in pigs and cattle.

As far as expression of the integrated foreign genes is concerned, it has been shown in mice that a high degree of appropriate expression can be achieved when certain cis-acting elements are contained in the foreign gene construct, so that approximately 90% to 100% of the Mt-I-GH transgenic mice express the foreign gene (52,55). In pigs this expression frequency is 50% and in rabbits 25% although the same Mt-I-hGH fusion genes have been transferred (114). It should be emphasized that results taken from one species such as the mouse can be considered to apply to other species only to a limited extent. This appears also to be true for results from molecular biology, as cis-acting elements obviously are controlled differently by varying trans-acting factors in genomes of different species. Therefore, transgenic mouse work alone is only of limited value when problems arising in the production of transgenic livestock are to be solved, particularly when there is a necessity of finding cis-acting factors which are suitable

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The production of foreign growth hormone (GH) in transgenic rabbits and pigs has not shown any phenotypic effects in terms of increased growth rates thus far (124). The reasons for this are not understood. Thus, further studies on the developmental regulation and functional effect of endogenous as well as foreign gene products in livestock species are required. It might well be that the inefficiency of foreign GH produced continuously in Mt-I-GH transgenic pigs is due to refractoriness of corresponding hormone receptors on the target cells caused by the unphysiological early onset of GH expression during embryonic development. Experiments with different promoters which lead to regulated expression only after birth are under way in our laboratory and will enable us to answer this question.

Future Impact on Livestock Production

The many advantages of transgenic livestock production have been discussed extensively elsewhere (128,129). These include improvement of productivity as well as the transfer of disease resistance in farm animals. Alteration of cell surface antigens which has been possible in transgenic mice (96,97), for example, can possibly cause resistance of transgenic animals towards certain species-specific viruses. Herpes virus infections, causing diseases such as infectious bovine rhinotracheitis or pseudorabies in pigs are especially challenging as immunization against these viruses is highly ineffective under field conditions. Also, overall disease resistance could be increased if stimulation of unspecific resistance factors such as interferon, for example, could be achieved via gene transfer.

Current work is mainly concentrating on farm animals which have integrated foreign GH fusion genes to increase their growth rate and therefore their productivity. In cattle this gene could also be used to increase milk production when fused to lactation-specific promotors. In this context attention should be paid to the fact that highly productive farm animals which have been bred during the last centuries with mainly phenotypical selection methods are biochemically extremely balanced organisms and require more than one gene for high productivity, especially when parameters as fertility and longevity are also desired, as for instance, in dairy cattle. Generally, a negative correlation between high productivity parameters and overall health of farm animals has been observed, but there are individual exceptions.

It should be emphasized that further progress in research of the molecular genetics of farm animals will provide the information necessary not only to introduce new genes into the genome but also to contra-select for certain genes which interfere with healthy performance. This could even be done *in vitro* if totipotent cell lines of livestock were available, thus transferring

directed mutation and subsequent selection mainly to in vitro systems in order to establish phenotypically healthy and highly productive livestock breeds.

Since the work in mice has shown that far more is possible today in respect to genotype alteration than had been assumed five years ago, there is a high probability that the production of genetically improved livestock by gene transfer and in vitro selection will also be feasible. However, a large amount of scientific research needs to be done in livestock species requiring the cooperation of embryologists, molecular biologists and animal physiologists before this can be achieved.

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PLANT REPORTER GENES:

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